BLOOD RESEARCH
VOLUME 51 · NUMBER 1
March 2016
ORIGINAL ARTICLE

Influence of genetic polymorphisms in the folate pathway on toxicity after high-dose methotrexate treatment in pediatric osteosarcoma

Jeong A Park[1], Hee Young Shin[2]

[1]*Department of Pediatrics, Haeundae Paik Hospital, Inje University College of Medicine, Busan,* [2]*Department of Pediatrics, Cancer Research Institute, Seoul National University Children's Hospital, Seoul National University College of Medicine, Seoul, Korea*

p-ISSN 2287-979X / e-ISSN 2288-0011
http://dx.doi.org/10.5045/br.2016.51.1.50
Blood Res 2016;51:50-7.

Received on January 29, 2016
Revised on March 14, 2016
Accepted on March 22, 2016

*This research was supported by a grant from the Alumni of Pediatrics, Seoul National University Children's Hospital, Seoul, Korea.

Correspondence to
Hee Young Shin, M.D., Ph.D.
Department of Pediatrics, Cancer Research Institute, Seoul National University Children's Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea
E-mail: hyshin@snu.ac.kr

Background
Methotrexate (MTX), one of the main drugs used to treat osteosarcoma, is a representative folic acid antagonist. Polymorphisms of various enzymes involved in the metabolism of MTX could contribute to differences in response to MTX in pediatric osteosarcoma patients.

Methods
Blood and tissue samples were obtained from 37 pediatric osteosarcoma patients who were treated with high-dose MTX therapy. The following 4 single nucleotide polymorphisms (SNPs) were analyzed: *ATIC* 347C>G, *MTHFR* 677C>T, *MTHFR* 1298A>C and *SLC19A1* 80G>A. Serial plasma MTX concentrations after high-dose MTX therapy and MTX-induced toxicities were evaluated. Correlations among polymorphisms, MTX concentrations and treatment-induced toxicities were assessed.

Results
Plasma MTX levels at 48 hours after high-dose MTX infusion were significantly associated with *SLC19A1* 80G>A ($P=0.031$). Higher plasma levels of MTX at 48 and 72 hours were significantly associated with MTX-induced mucositis ($P=0.007$ and $P=0.046$) and renal toxicity ($P=0.002$), respectively. SNP of *SLC19A1* gene was associated with development of severe mucositis ($P=0.026$).

Conclusion
This study suggests that plasma levels of MTX are associated with GI and renal toxicities after high-dose MTX therapy, and genetic polymorphisms that affect the metabolism of MTX may influence drug concentrations and development of significant side effects in pediatric patients treated with high-dose MTX.

Key Words Pediatric, Osteosarcoma, Methotrexate, Toxicity, Single nucleotide polymorphism

INTRODUCTION

High-dose methotrexate (MTX) with leucovorin (5-formyltetrahydrofolic acid) rescue in combination with doxorubicin and a platinum agent has served as a cornerstone of neoadjuvant chemotherapy for osteosarcoma. High-dose MTX treatment is associated with various toxicities, including toxicities of central nervous system (CNS), liver, kidney, bone marrow and gastrointestinal system, particularly oral mucosa. Various studies have reported that several pharmacokinetic parameters, including a high plasma MTX concentration and prolonged exposure to high levels of MTX, were associated with the development of high-dose MTX-induced toxicities [1-3]. However, MTX levels exhibit significant inter-individual variability, and acute toxicity after high-dose MTX is often unexpected and not typically dose dependent; thus, it is difficult to predict who will develop a more serious adverse response to high-dose MTX. Folate pathway genes are involved in the metabolism of MTX and are very polymorphic. Numerous pharmacogenetic studies have reported that single nucleotide polymorphisms (SNPs) alter the activity or expression of folate pathway enzymes, which may influence the response to and toxicity caused by MTX

in various malignancies and autoimmune diseases [4-6].

However, comparatively few data are available regarding associations between various genetic polymorphisms of the folate pathway genes and pediatric osteosarcoma. We hypothesized that polymorphisms of the folate pathway genes may influence plasma concentrations of MTX and MTX-induced toxicity in pediatric patients with osteosarcoma. Among various candidate SNPs in folate pathway genes, SNPs in solute carrier family 19, member1 (*SLC19A1*), methylenetetrahydrofolate reductase (*MTHFR*) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (*ATIC*) are potential biomarkers that confer susceptibility to MTX. In this study, we retrospectively examined whether *SLC19A1* 80G>A, *MTHFR* 677C>T, *MTHFR* 1298A>C, and *ATIC* 347C>G polymorphisms affected plasma MTX concentrations and susceptibility to high-dose MTX-induced toxicity in Korean pediatric patients with osteosarcoma.

MATERIALS AND METHODS

Study population

The patients included in this study were Korean children with osteosarcoma who were treated with high-dose MTX-containing chemotherapeutic protocols between 1986 and 2010. This study was approved by the Ethics Committee of Institutional Review Board (IRB No. H-0906-067-284). In all cases, informed consent was obtained from the patients, their parents or both. The patients received high-dose MTX according to the following treatment protocols: CCG 7921A, CCG 7921B and COG AOST 0331 [7, 8]. High-dose MTX at 12 g/m^2 was administered separately by an interval of 1 week. Each infusion lasted for 4 hours. Intravenous hydration and alkalization were achieved 12 hours prior to the start of MTX therapy. High-dose MTX was started at a urine pH>7.0 to provide protection against MTX-induced renal dysfunction. Leucovorin rescue was initiated 24 hours after the start of MTX infusion at a dose rate of 15 mg/m^2, and the dosages were adjusted based on plasma MTX concentrations and continued until plasma MTX levels reached <0.1 μmol/L. The patient characteristics and clinical data used in this study were collected retrospectively.

MTX concentration and toxicity

Therapeutic drug monitoring was performed at 24, 48 and 72 hours from the beginning of the infusion of high-dose MTX. Plasma MTX concentrations were measured by a fluorescence polarization immunoassay on a TDx system (Abbot Laboratories, Abbot Park, IL). The highest plasma levels of total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine were used as criteria for liver and kidney toxicity. Grades of mucositis, liver, kidney and neurologic toxicities were assessed according to the CTCAE 3.0 [9].

DNA extraction and genotyping

Genomic DNA from peripheral blood and formalin-fixed, paraffin-embedded (FFPE) tumor tissues from the 37 patients were analyzed. Genomic DNA was extracted from peripheral blood lymphocytes and tumor tissues using a QIAamp DNA Mini Kit and a QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany), respectively, according to the manufacturer's instructions. DNA yield, integrity and protein contamination were determined by spectrophotometry. Candidate SNPs of genes involved in the folate pathway and the transport of MTX included *SLC19A1* 80G>A, *MTHFR* 677C>T and 1298A>C, and *ATIC* 347C>G.

Gene amplification

PCR amplification was performed in a total volume of 20 μL with a mixture containing 5 μM forward and reverse sequencing by synthesis (SBS) primers (Table 1), 0.25 mM deoxynucleotide (dNTP) mix, 112.5 mM Tris HCl (pH 9.0), 3 mM $MgCl_2$, 75 mM KCl, 30 mM $(NH_4)_2SO_4$, and 1.5 U of Taq polymerase (Biotools, Madrid, Spain). The reactions were preheated to 94°C for 2 minutes, followed by 10 amplification cycles, which consisted of the following: denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute. This protocol was followed by 40 amplification cycles using following parameters: denaturation at 94°C for 15 seconds, annealing at 60°C for 230 seconds and extension at 72°C for 40 seconds in a 96-well thermal cycler (Applied Biosystems, CA, USA). Sixteen microliters of PCR product was mixed with 4 μL of an activation solution (YeBT, Seoul, Korea), and the resultant mixture was incubated at 37°C for 60 minutes and 85°C for 15 minutes.

Table 1. Sequencing by synthesis (SBS) primer sequences for multiple amplification of methotrexate related genes.

Gene	Position	Sequence (5′ to 3′)
SLC19A1 c.80	Forward	GTGGAACCTGGG AAAA TGACCCCGAGCTCC
	Reverse	TGATGAAGCTCTC AAAA CTGGCCGTATCTGC
MTHFR c.677	Forward	CTGTCATCCCTATT AAAA CAGGTTACCCCAAAG
	Reverse	GTGCATGCCTTC AAAA AAAGCGGAAGAATGTG
MTHFR c.1298	Forward	CCTTTGGGGA AAAA TGAAGGACTACTACC
	Reverse	GCAAGTCACTTTGT AAAA CCATTCCGGTTTGG
ATIC c.347	Forward	TTTAATGACAGCCAGAAAAACGTCTTTGTTTTATAGA
	Reverse	AATCCCAAAACACAATAAAAAGAAGTAGCTATTTTCT

Table 2. Allele-specific primer extension (ASPE) primer sequences.

Gene	Taq sequence	Sequence (5′ to 3′)
SLC19A1 80G	TCAAAATCTCAAATACTCAAATCA	AAAGGTAGCACACGAGGC
SLC19A1 80A	CAATTCAAATCACAATAATCAATC	AAAGGTAGCACACGAGGT
MTHFR 677C	CTAACTAACAATAATCTAACTAAC	TGCGTGATGATGAAATCGG
MTHFR 677T	AATCTTACTACAAATCCTTTCTTT	TGCGTGATGATGAAATCGA
MTHFR 1298A	CTACAAACAAACAAACATTATCAA	GAGCTGACCAGTGAAGA
MTHFR 1298C	CAATATACCAATATCATCATTTAC	GAGCTGACCAGTGAAGC
ATIC 347C	TCAATTACCTTTTCAATACAATAC	CACAGCCTCCTCAACAG
ATIC 347G	TTACTCAAAATCTACACTTTTTCA	CACAGCCTCCTCAACAC

Labeling of activation products

Five μL of activated PCR product was added to 20 μL of an allele-specific primer extension (ASPE) reaction mixture containing 75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 25 nM ASPE primer mix (Table 2), 6 mM biotin dCTP, 50 mM of dATP, dGTP, dTTP mix and 1.0 U of Taq polymerase (Biotools, Spain). The reactions were denatured at 94°C for 5 minutes, followed by 35 amplification cycles to label the amplicons: 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 7 minutes.

Hybridization of amplicons

Microspheres (FlexMAP beads, Tm Bioscience, Toronto, Canada) with anti-tags for each allele-specific primer were added to 22 μL of the ASPE reaction product to a final volume of 42 μL in 2× Hybrisol (YeBT, Seoul, Korea). The mixtures were denatured for 10 min at 95°C, and then incubated for 30 min at 37°C. The microspheres were washed thrice in 160 μL of TM hybridization buffer (0.2 M NaCl, 0.1 M Tris (pH 8.0), 0.08% Triton X-100). Streptavidin-R-phycoerythrin (SAPE, MOSS, Pasadena, MD) was diluted 1:500 in TM hybridization buffer, and 100 μL was added to the microsphere-hybridized ASPE reaction products. The mixture was incubated for 15 min at room temperature.

Data analysis

Microsphere fluorescence was measured using a Luminex 200 cytometer (Luminex, Austin, TX). Data were collected from a minimum of 50 microspheres of each type. Masterplex GT software (Miraibio, San Francisco, CA) was used to analyze SNP genotypes. Homozygous alleles were discriminated by differences of 25% in median fluorescence intensity (MFI). The difference ratio was calculated by dividing the net MFI of one allele by the sum of the net MFI of all alleles and multiplying by 100.

Statistical analysis

The associations between SNPs and plasma MTX concentrations at 24, 48, and 72 hours after high-dose MTX infusion were evaluated using one way ANOVA, Kruskal-Wallis test and Mann-Whitney test. Analyses of plasma MTX concentrations and MTX-induced toxicities were performed using Mann-Whitney test. The frequencies of severe complications among SNPs were compared with a chi-square test and Fisher's exact test. *P*-values less than 0.05 were considered as statistically significant. All statistical analyses were performed using SPSS version 22 (SPSS, Inc., Chicago, IL, USA).

Table 3. Characteristics of patients.

Characteristics	No. of Patients
Total number of patients	37
Age at diagnosis (yr)	7.2–15.7 (median 11.8)
Gender (male/female)	21/17
Primary site	
Femur	18 (48.6%)
Tibia/fibula	10 (27.0%)
Humerus/radius	6 (16.2%)
Axial	3 (8.1%)
Metastasis at diagnosis	
Absent	24 (64.9%)
Present	13 (35.1%)
Histological subtype	
Osteoblastic	32 (86.5%)
Chondroblastic	3 (8.1%)
Telangiectatic	2 (5.4%)
Total number of high-dose MTX therapy	393
No. of high-dose MTX therapy per person	2–20 (median 10)

RESULTS

A total of 37 pediatric patients with osteosarcoma were enrolled in this study. Table 3 presents the characteristics of the patients. The median age was 11.8 years, and the femur was the most common site of primary tumor. One-third of the patients had distant metastasis at diagnosis. Each patient received a median of 10 cycles (range, 2–20) of high-dose MTX therapy. For some patients high-dose MTX was associated with serious toxicities resulting in treatment interruption or discontinuation. A total of 393 courses of high-dose MTX chemotherapy were evaluated in this study.

Plasma MTX concentration and genetic polymorphism

The distribution of the examined folate pathway gene

SNPs is listed in Table 4. These SNPs consisted of common polymorphisms in *SLC19A1* (G80A, rs1051266), *MTHFR* (C677T, rs1801133; A1298C, rs1801131), and *ATIC* (C347G, rs2372536). Plasma MTX concentrations were measured at 24, 48 and 72 hours from the start of infusion of high-dose MTX. The median MTX concentrations were 4.7 μmol/L (range, 0.19–378.6 μmol/L) at 24 hours, 0.65 μmol/L (range, 0.09–97.3 μmol/L) at 48 hours, and 0.3 μmol/L (range, 0.04–9.75 μmol/L) at 72 hours. Whereas the 347C>G polymorphism of *ATIC* and the 677C>T and 1298A>C polymorphisms of *MTHFR* did not show any correlations with serial plasma MTX levels after high-dose MTX therapy, the 80G>A variants of *SLC19A1* had significantly lower plasma MTX levels at 48 hours (P=0.03), (Table 5 and Fig. 1).

Table 4. Genotype frequencies of the patients.

Polymorphism	Genotype	No. of patients (%)
ATIC 347C>G	CC	18 (48.6)
	CG	7 (18.9)
	GG	2 (5.4)
	N/A	10 (27.0)
MTHFR 677C>T	CC	9 (24.3)
	CT	18 (48.6)
	TT	8 (21.6)
	N/A	2 (5.4)
MTHFR 1298A>C	AA	21 (56.8)
	AC	15 (40.5)
	CC	1 (2.7)
SLC19A1 80G>A	GG	8 (21.6)
	GA	15 (40.5)
	AA	14 (37.8)

Plasma MTX concentration and MTX-induced toxicity

High-dose MTX-induced toxicity was recorded and graded

Table 5. Associations between SNPs and plasma methotrexate concentrations at 48 hours after high-dose methotrexate therapy.

Polymorphism	Genotype	No. of patients	MTX level, median (range)	*P*
ATIC 347C>G	CC	17	0.51 (0.09–7.5)	
	CG	7	0.65 (0.21–1.35)	0.971
	GG	2	0.94 (0.21–1.66)	
	CG/GG	9	0.65 (0.21–1.66)	0.833
MTHFR 677C>T	CC	8	0.62 (0.34–1.36)	
	CT	17	0.65 (0.09–97.3)	0.965
	TT	8	0.77 (0.15–4.98)	
	CT/TT	25	0.65 (0.09–97.3)	0.821
MTHFR 1298A>C	AA	20	0.78 (0.09–97.3)	
	AC	14	0.43 (0.13–7.5)	0.416
	CC	1	1.36	
	AC/CC	15	0.47 (0.13–7.5)	0.521
SLC19A1 80G>A	GG	8	1.26 (0.37–6.8)	
	GA	15	0.34 (0.13–1.92)	0.031
	AA	12	0.67 (0.09–97.3)	
	GA/AA	27	0.47 (0.09–97.3)	0.030

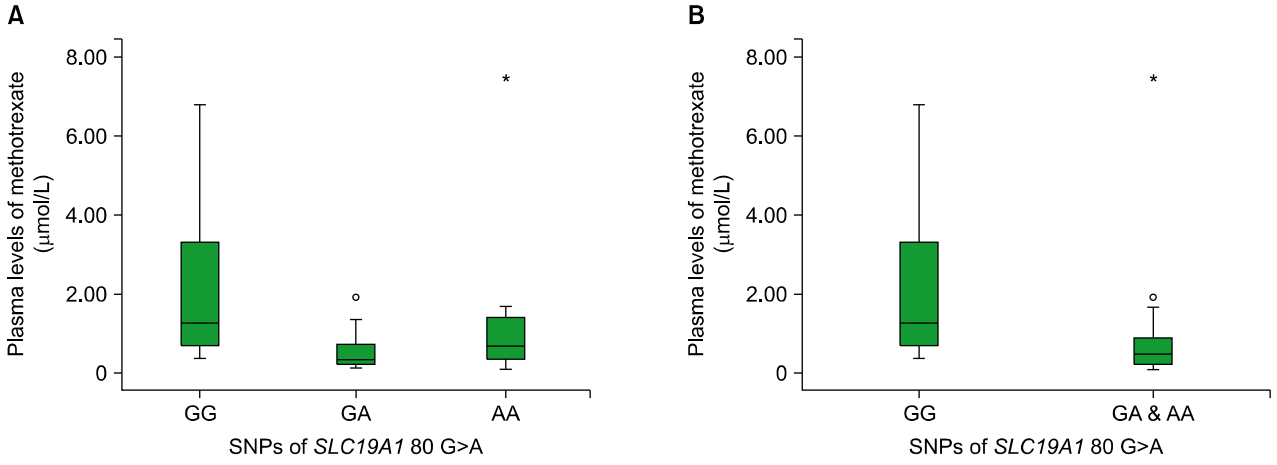

Fig. 1. Plasma methotrexate levels at 48 hours after high-dose methotrexate infusion were significantly associated with the 80G>A variants of *SLC19A1* (P=0.03).

according to Common Terminology Criteria for Adverse Events version (CTCAE) 3.0. At least one episode of grade 3–4 liver toxicity was experienced by 26 patients (70.3%). In addition, grade 3 to 4 kidney toxicity and neurotoxicity were observed in 4 and 1 patients (10.8% and 2.7%), respectively. Mucositis higher than grade 3 was experienced by 4 patients (10.8%). The results of the analysis on MTX-induced toxicity and plasma MTX concentrations at 48 and 72 hours after high-dose MTX infusion are displayed in Table 6. Severe liver, kidney, and neurologic toxicities and mucositis were as defined by the presence of grade 3 or higher symptoms. Higher plasma MTX levels at 48 hours were associated with severe mucositis and kidney toxicity (P=0.007 and P=0.002), and MTX levels at 72 hours also showed associations with development of severe mucositis and kidney toxicity (P=0.046 and P=0.002), (Fig. 2). Plasma MTX levels

Table 6. Analysis of MTX-induced toxicity and MTX concentrations at 48 and 72 hours after high-dose MTX therapy.

Toxicity		No. of patients	MTX level at 48 hr median (range)	*P*	MTX level at 72 hr median (range)	*P*
Liver	Grade ≤2	9	0.27 (0.13–1.36)	0.119	0.17 (0.04–0.61)	0.339
	Grade ≥3	26	0.66 (0.09–97.3)		0.20 (0.02–9.75)	
Kidney	Grade ≤2	31	0.51 (0.09–7.50)	0.002	0.19 (0.02–2.48)	0.002
	Grade ≥3	4	4.36 (1.35–97.3)		0.80 (0.64–9.75)	
CNS	Grade ≤2	34	0.60 (0.09–97.3)	0.4	0.20 (0.02–9.75)	0.45
	Grade ≥3	1	1.64		0.61	
Mucositis	Grade ≤2	31	0.51 (0.09–97.3)	0.007	0.19 (0.02–9.75)	0.046
	Grade ≥3	4	3.31 (1.17–7.50)		0.62 (0.20–2.48)	

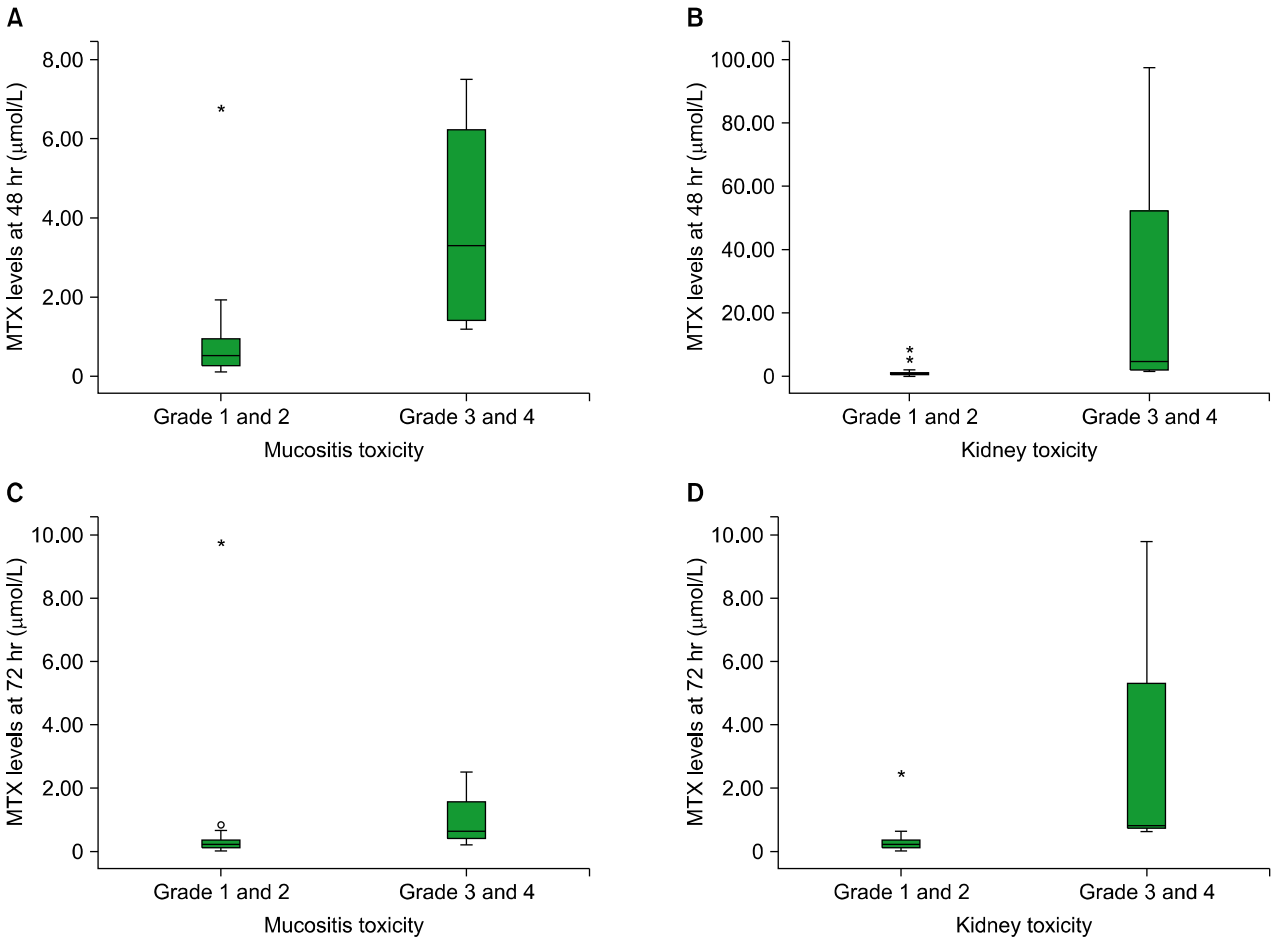

Fig. 2. Plasma methotrexate levels at 48 (**A, B**) and 72 hours (**C, D**) after high-dose methotrexate infusion were significantly associated with a development of grade 3 and 4 of mucositis and kidney toxicity.

Table 7. Analysis of SNPs and methotrexate-induced toxicity.

Gene	SNPs	Liver toxicity					Kidney toxicity					Mucositis				
		≤Grade 2 N (%)	≥Grade 3 N (%)	OR	*P*	95% CI	≤Grade 2 N (%)	≥Grade 3 N (%)	OR	*P*	95% CI	≤Grade 2 N (%)	≥Grade 3 N (%)	OR	*P*	95% CI
ATIC 347 (N=27)	CC	5 (28)	13 (72)				16 (89)	2 (11)		1.0		15 (83)	3 (17)			
	CG	4 (57)	3 (43)		0.30		6 (86)	1 (14)				7 (100)	0 (0)		0.63	
	GG	1 (50)	1 (50)				2 (100)	0 (0)				2 (100)	0 (0)			
	CG/GG	5 (56)	4 (44)	0.31	0.22	0.06–1.64	8 (89)	1 (11)	1.0	1.0	0.08–12.76	9 (100)	0 (0)	0.83	0.53	0.68–1.03
MTHFR 677 (N=35)	CC	3 (33)	6 (67)				9 (100)	0 (0)		0.152		9 (100)	0 (0)			
	CT	7 (39)	11 (61)		1.81		14 (78)	4 (22)				17 (94)	1 (6)		0.07	
	TT	1 (13)	7 (88)				8 (100)	0 (0)				5 (63)	3 (38)			
	CT/TT	8 (31)	18 (69)	1.13	1.00	0.22–5.67	22 (85)	4 (15)	1.18	0.553	1.00–1.39	22 (85)	4 (15)	1.18	0.55	1.00–1.39
MTHFR 1298 (N=37)	AA	5 (24)	16 (76)				18 (86)	3 (14)		0.67		18 (86)	3 (14)			
	AC	5 (33)	10 (67)		0.31		14 (93)	1 (7)				14 (93)	1 (7)		0.67	
	CC	1 (100)	0 (0)				1 (100)	0 (0)				1 (100)	0 (0)			
	AC/CC	6 (38)	10 (63)	0.52	0.48	0.13–2.17	15 (94)	1 (6)	0.4	0.62	0.04–4.26	15 (94)	1 (6)	0.40	0.62	0.04–4.26
SLC19A1 (N=37)	GG	1 (13)	7 (88)				7 (88)	1 (13)		1.0		5 (63)	3 (38)			
	GA	7 (47)	8 (53)		0.22		13 (87)	2 (13)				15 (100)	0 (0)		0.01	
	AA	3 (21)	11 (79)				13 (93)	1 (7)				13 (93)	1 (7)			
	GA/AA	10 (34)	19 (66)	0.27	0.39	0.03–2.53	26 (90)	3 (10)	0.81	1.0	0.07–9.01	28 (97)	1 (3)	0.06	0.03	0.01–0.69

at 24 hours after the high-dose MTX infusion did not show significant associations with MTX-induced toxicities.

Associations between genetic polymorphisms and high-dose MTX-induced toxicity

Results of the analysis about the associations between the candidate gene SNPs and development of grade 3 or 4 toxicities are described in Table 7. *SLC19A1* 80G>A had a lower risk of developing severe mucositis (OR, 0.06; *P*=0.026; 95% CI, 0.005–0.693). Any of the remaining SNPs did not show significant correlations with severe liver and kidney toxicity or mucositis.

DISCUSSION

Identifying genetic predictors of MTX-related toxicity may help to determine individual dosage adjustment and to minimize adverse effects. This study analyzed polymorphisms of several candidate genes involved in the folate-MTX metabolic pathway and investigated possible associations between these SNPs and clinical data of osteosarcoma patients after high-dose MTX therapy. In clinical practice, plasma MTX monitoring is essential after high-dose MTX infusion to detect those who are at risk for MTX-related toxicity and to determine the dose and duration of leucovorin rescue. Various cutoff points based on the MTX plasma half-life have been used to determine the rate of MTX clearance and leucovorin rescue to prevent or minimize toxicity. Studies evaluating plasma MTX concentrations at 24, 48, and 72 hours have concluded that significant effects were observed at 48 hours, and plasma MTX levels at 48 hours after high-dose MTX infusion were found to be independent of both treatment protocol and patient age [10-12].

In this study, MTX plasma levels at 48 hours after high-dose MTX therapy showed an association with the *SLC19A1* 80G>A polymorphism. Patients with the GG allele at the *SLC19A1* 80G>A polymorphism exhibited higher plasma MTX levels. SLC19A1, a major route for the transport of folates in mammalian cells, is an anion exchanger that transports both folate and methotrexate into cells. The *SLC19A1* 80G>A is associated with an Arg27His amino acid substitution. Some studies reported that higher MTX plasma levels were found for *SLC19A1* 80AA genotype [13], but others demonstrated no significant association with MTX plasma levels for *SLC19A1* G80A [11, 14]. Although a functional difference between the 80A allele and the 80G allele has yet to be determined, low expression or changes in the molecular structure, function, and activity of SLC19A1 can affect MTX transmembrane transport capacity and plasma and intracellular MTX concentrations [15, 16].

High-dose MTX therapy can cause significant side effects, including myelosuppression, mucositis, nausea, vomiting, diarrhea, hepatotoxicity, and reduced kidney function as well as neurotoxicity [17]. Prior to the routine monitoring of plasma MTX concentrations and dosing of leucovorin accordingly, the incidence of fatal toxicity after high-dose MTX reached approximately 5%, and early studies revealed that a high risk for bone marrow and gastrointestinal mucosal toxicities was related to MTX concentrations greater than 5 to10 μmol/L at 24 hours, 1 μmol/L at 48 hours, and 0.1 μmol/L at 72 hours [18-20]. However, it has become mandatory to monitor plasma MTX during high-dose MTX therapy to adjust hydration, alkalization, and leucovorin rescue individually. Thus, in most cases, these toxicities can be prevented and ameliorated by the administration of leucovorin [21, 22]. Nevertheless, this study showed that plasma MTX concentrations at 48 and 72 hours were significantly associated with MTX-related toxicity, especially mucositis and kidney toxicity.

Toxic response to high-dose MTX displays great inter-individual variability. Many studies have suggested that genetic

factors contribute to the occurrence of MTX-related toxicity and that MTX toxicity can be modified by SNPs in genes involved in the metabolism, transport, and functions of folates and MTX [23-25]. This study also identified that the *SLC19A1* 80G>A had a protective role against developing significant mucositis. There are several proposed mechanisms for MTX-induced mucositis. MTX may be secreted in the saliva, leading to increased direct mucosal toxicity, and causes alterations in glutathione metabolism, variations in gastrointestinal microflora, and variable inflammatory responses by TNF-α, IL-2, IL-6, and C-reactive protein [26, 27]. Variations in these proinflammatory cytokines and folate metabolic pathway genes have been suggested to play an important role in MTX-induced mucositis [28, 29]. The *SLC19A1* gene is located on chromosome 21, which explains the increased MTX sensitivity in children with high hyperdiploidy (which nearly includes trisomy 21) or Down syndrome [30]. Although functional studies of the *SLC19A1* 80G>A SNP have led to somewhat ambiguous results, the amino acid change in the first transmembrane domain of SLC19A1 caused by the G>A substitution alters its transport capacity and the ratio of SLC19A1 affinity to MTX versus other folate substrates. Carriers of the 80A allele are assumed to have higher affinity for other folate substrates [13, 24]. This also explains our results showing that homozygous carriers of the GG allele had a significantly increased risk of severe mucositis. Regarding gastrointestinal toxicity, Shimasaki *et al.* reported that serious vomiting episodes, as defined by the presence of grade 2 symptoms or worse, occurred more frequently in individuals with an increasing number of G alleles [11]. Beyond the cause of lower MTX plasma level, with leucovorin rescue after high-dose MTX therapy, the *SLC19A1* 80G>A polymorphism might impart a protective role against severe MTX-induced mucositis.

In conclusion, our study demonstrated the associations between MTX plasma levels at 48 and 72 hours after MTX infusion and renal toxicity and mucositis. In addition, we identified the influence of *SLC19A1* 80G>A on high-dose MTX-related toxicity and plasma levels. Polymorphisms of *SLC19A1* 80G>A need to be further investigated as this result suggested that they may have significant roles in MTX toxicity and in increased plasma MTX level. Identification of genetic variants that contribute to serious toxicities would be the first step toward developing predictive diagnostic markers that reduce the incidence of severe toxicities and improve treatment outcomes. Individual dose adjustment based partly on genetic predisposition holds promise as a strategy to minimize the risk of serious toxicity without compromising efficacy.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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