Original Article

Relationship between methylenetetrahydrofolate reductase gene polymorphisms and methotrexate drug metabolism and toxicity

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Background: Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, and methotrexate (MTX) is the key drug for ALL. Studies on the relationship between High-Dose methotrexate (HD-MTX) toxicity and methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C genes have drawn different conclusions. This study aimed to investigate the relationship between the polymorphism of *MTHFR* C677T and A1298C genes and the toxicity responses of MTX.

Methods: The *MTHFR* C677T and A1298C genotypes of 271 children with ALL who received HD-MTX chemotherapy in southern China from September 2017 to June 2021 were analyzed, and the toxicity of HD-MTX was evaluated and analyzed according to Common Terminology Criteria for Adverse Events (CTCAE) 5.0.

Results: The *MTHFR* C677T and A1298C gene polymorphisms were not correlated with the 48-hour MTX blood concentrations (P>0.05). Unconditional logistic regression model analysis also revealed that the risk of liver function impairment [odds ratio (OR) =1.656, 95% confidence interval (CI): 1.179–2.324, P<0.05] and mucosal damage (OR =1.508, 95% CI: 1.042–2.183, P<0.05) were 1.656 and 1.508 times higher for the heterozygous mutant (CT), and homozygous mutant (TT) mutant type than for the wild-type (CC), wild-type, respectively. The risk of neutropenia and liver function impairment were 0.498 (OR =0.498, 95% CI: 0.251–0.989, P<0.05) and 6.067 (OR =6.067, 95% CI: 1.183–31.102, P<0.05) times higher in low-risk children with CT+TT mutant genotypes than in those with CC wild genotypes, respectively. Furthermore, the risk of mucosal damage was 1.906 times higher in high-risk children with the CT+TT genotype than in those with the CC genotype (OR =1.906, 95% CI: 1.033–3.518, P<0.05). The *MTHFR* A1298C genotypes differed in the incidence of liver function damage and gastrointestinal toxic reactions in children with ALL. Nonetheless, no increased risk of liver function impairment nor gastrointestinal reactions in children with the heterozygous mutant (AC)+CC mutation was observed.

Conclusions: Advancements in *MTHFR* genotype testing in children with ALL and the introduction of personalised treatments based on genotype results during HD-MTX chemotherapy will help to predict, prevent, and reduce the occurrence of adverse MTX-related toxic reactions.

Keywords: Acute lymphoblastic leukaemia; methotrexate; methylenetetrahydrofolate reductase; gene polymorphism; toxic reaction

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Introduction

Leukemia is the most common malignancy in children, accounting for approximately 40% of all childhood malignancies, and is one of the leading causes of cancer-related deaths in childhood. Moreover, approximately 80% of all leukemia cases in children are acute lymphoblastic leukemia (ALL) (1). ALL, a malignancy originating from hematopoietic stem cells of B or T lymphocyte precursors, is driven by a series of genetic aberrations, including mutations, chromosomal translocations, and the involvement of aneuploidy genes in lymphocyte development and cell cycle regulation (2). Advances in treatment techniques have increased the 5-year survival rates of more than 90% of children with ALL over the past 20 years (3). However, chemotherapy-related toxicities, which may lead to drug underdosing, and treatment interruption or discontinuation remain major factors affecting treatment outcomes and warrant detailed investigation (4).

The administration of methotrexate (MTX), a key drug in systemic intensive therapy for ALL (5), has been well established in the consolidation phase of treatment and in the prevention and management of extramedullary leukemia in clinical practice. It is also important for sustained remission, relapse prevention, and improvement of long-term disease-free survival in childhood ALL. However, high-dose administration of MTX can cause various adverse effects, including myelosuppression, mucosal damage, gastrointestinal reactions, hepatic and renal toxicity, and neurotoxicity, which eventually lead to forced dose reduction or discontinuation of chemotherapy (6,7). Based on clinical reports, the same dose of MTX used in different children can exhibit varying severities of adverse reactions, indicating different sensitivity and tolerance levels and high MTX heterogeneity in different individuals (8). Individual genetic polymorphisms (especially single nucleotide polymorphisms) have a highly variable effect on the metabolism of MTX, which could be the main reason for the varying levels of drug sensitivity and tolerance (9).

MTX is an intracellular folate analogue that is actively transported into the cell via reduced folate carrier-1. It inhibits thymidylate synthase to interfere with cellular DNA synthesis (10). MTX also blocks the folate cycle by inhibiting dihydrofolate reductase and affects other important enzymes in the folate pathway, such as methylenetetrahydrofolate reductase (MTHFR). *MTHFR* is a key enzyme in intracellular folate dynamic homeostasis and metabolism, and catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, mediates DNA methylation, and affects protein synthesis (11). Moreover, structural or functional abnormalities in MTHFR can interfere with nucleic acid synthesis and induce cell death. Additionally, it can also affect cellular responsiveness to MTX and the toxic response to MTX. Therefore, *MTHFR* is considered a key therapeutic target of MTX and its metabolites (10,12,13).

The *MTHFR* gene is located on the short arm of the distal region of chromosome 1 (1p36.3), spanning 2.2 kb in length and comprising 11 exons. While numerous polymorphisms of *MTHFR* have been reported, to date, only two polymorphisms, C677T and A1298C, have been extensively studied. The polymorphism of C677T and A1298C gene can reduce the stability and enzyme activity of MTHFR, thus affecting MTX metabolism. The mutational status of gene polymorphisms at these loci could lead to altered *MTHFR* activity and thus affect MTX metabolism. Therefore, an in-depth understanding of single nucleotide polymorphism (SNP) loci in the *MTHFR* gene may help to reduce the severe toxic reactions induced by HD-MTX exposure in children with ALL, and facilitate the optimization and individualization of MTX therapy. This current study investigated the effects of the *MTHFR* C677T and A1298C gene polymorphisms on MTX metabolism and related toxicity during childhood ALL treatment. And we collect the genotype distribution of the C677T and A1298C loci in the *MTHFR* gene of some ALL patients before

Highlight box

Key findings

- The study found the *MTHFR* C677T gene polymorphism increases the risk of liver toxicity and mucosal damage in children with ALL treated with HD-MTX regimens.

What is known and what is new?

- Functional variants of the *MTHFR* gene may affect the efficacy and toxic alterations of MTX. Currently, the relationship between *MTHFR* C677T and A1298C gene polymorphisms and MTX toxic response has been extensively studied, definitive conclusions and uniform consensus remain unavailable.
- The present study, as a multicenter retrospective study with large sample size provides additional evidence for the relationship between MTX toxic response and *MTHFR* C677T and A1298C gene polymorphisms in children with ALL from southern China.

What is the implication, and what should change now?

- In subsequent studies, the study population size should be expanded, more genes and polymorphic loci related to MTX metabolism should be included.

treatment. This data will contribute to the development of individualised treatment regimens. We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-671/rc).

Methods

General information

Children with ALL who were diagnosed using the the MICM diagnostic model, treated according to the South China Children's Leukemia Group (SCCLG)-ALL-2016 protocol, and had completed at least 1 cycle of consolidation chemotherapy between September 2017 and June 2021 in southern China (Sun Yat-sen Memorial Hospital of Sun Yat-sen University, The Third Affiliated Hospital of Sun Yat-sen University, The First Affiliated Hospital of Sun Yat-sen University, Shenzhen Children's Hospital, and Sun Yat-sen University Cancer Center) were enrolled in this study. Informed consent was obtained from patients' legal guardians, and this study was approved by the Ethics Committee of The Third Affiliated Hospital, Sun Yat-sen University (No. (2022)02-076-01). The other hospitals are informed and agreed with the study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Patients were stratified into 3 groups—low-risk, intermediate risk and high risk—according to the age of onset, peripheral blood leukocyte counts since onset, immune classification (B ALL or T ALL), fusion genes, central nervous system leukemia, testicular lymphoblast invasion, mediastinal invasion and the response to 7-day prednisone treatment and remission on Day 15 and Day 33. Routine blood, liver, and kidney functions were analyzed prior to chemotherapy. All patients met the requirements for consolidation chemotherapy administration, and exhibited no gastrointestinal discomfort such as nausea and vomiting, fever, nor skin mucosal breakage. Additionally, all participants were in complete bone marrow remission [minimal residual disease (MRD) <0.01%] after VDLD (Vincristine + Dexamethasone + L-Asparaginase + Daunorubicin)-induced remission treatment.

Chemotherapy regimens

Based on the consolidation phase of the SCCLG-2016-ALL protocol, patients in the low-risk group received 2 g/m^2 MTX while the intermediate- and high-risk groups were treated with 5 g/m^2 MTX, which was administered via a 24-hour continuous intravenous drip. In detail, one tenth of the total dose was administered as a loading dose over 30 minutes. This was followed by lumbar puncture and one triple (MTX + Cytarabine + Dexamethasone) intrathecal injection and the remaining MTX was titrated uniformly over the next 23.5 hours. High-dose hydration and alkalinization (total fluid 3,000 mL/m^2·d, total alkaline 5 mL/kg·d) were performed at least 4 hour before and 72 hour after HD-MTX chemotherapy, wherein urinary pH ≥7.0 was maintained. Calcium formyl tetrahydrofolate (CF) 15 mg/m^2 was administered every 6 hours, for 36–42 hours after MTX treatment initiation. Additionally, serum MTX blood concentrations were measured at 24 and 48 hours, and the dose of CF was adjusted as required. Resuscitation was terminated when the serum MTX concentration was <0.2 μmol/L. Post-chemotherapy procedures included mouth rinsing, as well as anti-vomiting and stomach protection regimens. Routine blood and urine concentrations, liver and kidney function indexes, skin mucosal changes, nausea, vomiting, diarrhea, and other gastrointestinal reactions were monitored from chemotherapy initiation to 7 days after chemotherapy completion.

Genotyping

A total of 3 mL of peripheral blood was collected from the participants in EDTA anticoagulation tubes for DNA extraction before MTX chemotherapy. The samples were genotyped using polymerase chain reaction-chip hybridization to obtain the genotype distribution of the C677T and A1298C loci in the *MTHFR* gene.

Monitoring MTX blood concentrations

The MTX blood concentration was measured by collecting 2 ml of peripheral blood and separating the serum at 24 hours and 48 hours after HD-MTX administration. Blood concentrations >0.2 μmol/L 48 hours after MTX infusion was defined as high MTX concentration, whereas blood concentrations ≤0.2 μmol/L 48 hours after MTX infusion was defined as low MTX concentration.

Evaluation of toxicity

Toxic reactions that occurred during HD-MTX

Table 1 Grading criteria for high-dose methotrexate toxic reactions (CTCAE 5.0)

Toxic and side effects	Grade 0	Grade I	Grade II	Grade III	Grade IV
WBC ($\times10^9$/L)	≥4.0	3.0–3.9	2.0–2.9	1.0–1.9	<1.0
N ($\times10^9$/L)	≥2.0	1.5–1.9	1.0–1.4	0.5–0.9	<0.5
Hb (g/L)	≥110	95–109	80–94	65–79	<65
PLT ($\times10^9$/L)	≥100	75–99	50–74	25–49	<25
ALT (ALT*N)	≤1.5	1.5–3.0	3.1–5.0	5.1–20.0	>20.0
SCR (SCR*N)	≤1.0	1.0–1.5	1.6–3.0	3.1–6.0	>6.0
Oral mucosal damage	No	Erythema and pain	Ulcers that do not interfere with eating	Ulcers resulting in only fluids diet	Difficulty in eating
Nausea	No	Loss of appetite	Reduced oral intake	Need for nasal feeding or parenteral nutrition	
Vomiting	No	No intervention required	Additional intravenous rehydration required	Need for tube feeding or parenteral nutrition	
Diarrhea	No	Increased number: <4 times/day	Increased number: 4–6 times/day	Increased number: ≥6 times/day	

WBC, white blood cell count; N, neutrophil count; Hb, hemoglobin; PLT, platelet count; ALT, alanine transaminase; SCR, serum creatinine.

chemotherapy were recorded, including bone marrow suppression, liver toxicity, kidney toxicity, gastrointestinal reactions, and mucosal damage. The above toxic reactions were classified as grades 0, I, II, III, and IV according to the Common Terminology Criteria for Adverse Events (CTCAE) 5.0. Toxic reactions ≥ grade II indicated clinically relevant adverse chemotherapy reactions (*Table 1*).

Statistical analysis

Data analyses were performed using the IBM SPSS 25.0 statistical software. The allele frequencies of *MTHFR* C677T and A1298C were calculated, and the cardinality test was used to determine whether the Hardy-Weinberg equilibrium was met. Additionally, the cardinality test also compared the differences in MTX blood concentrations between the different genotypes of *MTHFR* C677T and A1298C at 48 hours after HD-MTX chemotherapy. A chi-square test compared the differences in the occurrence of toxic reactions after HD-MTX chemotherapy between *MTHFR* C677T and A1298C genotypes. The unconditional logistic regression model was used to calculate the odds ratio (OR) and 95% confidence interval (95% CI) for the likelihood of toxic reactions in individuals with different genotypes. Unless otherwise stated, $P<0.05$ was considered statistically significant.

Results

A total of 271 children with ALL were included in this study, including 158 (58.3%) males and 113 (41.7%) females, with an age range of 0.9 to 15.4 years and a median age of 5.9 years. Children were classified into low-risk (38 cases, 14.0%), intermediate-risk (172 cases, 63.5%), and high-risk (61 cases, 22.5%) groups based on the risk stratification criteria of the SCCLG-ALL-2016 protocol. The immunophenotype was predominantly B-cell type (242/271, 89.3%), while T-cell type accounted for only 10.7% (29/271) (*Table 2*). Complete experimental data from 32 participants could not be obtained for the four cycles of the mM regimen due to incomplete treatment or transfer to an external hospital. Additionally, data from 169 MTX treatment dosage adjustments due to previously high MTX blood levels, serious adverse events or renal charts suggesting poor renal metabolism were excluded, resulting in a total of 918 HD-MTX treatment sessions that were included in this study. Therapeutic concentrations of 24-hour MTX blood levels were achieved in all 918 sessions. Of these, 402 sessions (43.9%) achieved 48-hour MTX blood concentrations ≤0.2 μmol/L, whereas 513 sessions (56.1%) achieved 48-hour MTX blood concentrations >0.2 μmol/L. All 271 children were tested for the *MTHFR* C677T genotype, with 55.0%, 36.9%, and 8.1% carrying the wild-type (CC), heterozygous mutant (CT), and pure

Table 2 Clinical characteristics of the study subjects

Clinical characteristics	Number of children with ALL	Number of MTX treatment sessions
Total	271	918
Sex		
Male	158 (58.3%)	525 (57.2%)
Female	113 (41.7%)	393 (42.8%)
Immunophenotyping		
B-cell	242 (89.3%)	819 (89.2%)
T-cell	29 (10.7%)	99 (10.8%)
Degree of risk		
Low risk	38 (14.0%)	141 (15.4%)
Intermediate risk	172 (63.5%)	597 (65.0%)
High risk	61 (22.5%)	180 (19.6%)
MTX dose		
2 g/m^2	38 (14.0%)	141 (15.4%)
5 g/m^2	233 (86.0%)	777 (84.6%)
C677T genotype		
CC[1] wild type	149 (55.0%)	488 (53.2%)
CT+TT mutant type	122 (45.0%)	430 (46.8%)
CT heterozygous mutation	100 (36.9%)	349 (38.0%)
TT homozygous mutation	22 (8.1%)	81 (8.8%)
A1298C genotype		
Undetected	198	637
AA wild type	42 (57.5%)	163 (58.0%)
AC+CC[2] mutant type	31 (42.5%)	118 (42.2%)
AC heterozygous mutation	25 (34.3%)	96 (34.2%)
CC homozygous mutation	6 (8.2%)	22 (7.8%)
48-h MTX blood concentration		
>0.2 μmol/L		513 (56.1%)
≤0.2 μmol/L		402 (43.9%)

ALL, acute lymphoblastic leukemia; MTX, methotrexate; CC[1], wild-type; CT, heterozygous mutant; TT, homozygous mutant; AA, wild-type; AC, heterozygous mutant; CC[2], homozygous mutant.

mutant (TT) genes, respectively. The genotype distribution was in accordance with the Hardy-Weinberg equilibrium (χ^2=0.875, P=0.349) in the study population. However, only 73 children were tested for the A1298C genotype due to differences in testing programs by the hospitals, with 57.5%, 34.3%, and 8.2% of patients carrying the wild-type (AA), heterozygous mutant (AC) and pure mutant (CC) genes, respectively, per the Hardy-Weinberg equilibrium (χ^2=0.3725, P=0.83).

MTX-related toxic reactions

A total of 918 HD-MTX sessions were included from 271 children with ALL. The most common toxic reaction was myelosuppression, with 627 (68.3%), 481 (52.4%), 594 (64.7%), and 36 (3.9%) sessions of grade II or higher leukopenia, neutropenia, hemoglobin reduction, and thrombocytopenia, respectively. Additionally, 193 (21.0%), 71 (7.73%), and 133 (14.5%) episodes of grade II or higher hepatotoxicity, gastrointestinal reactions, and mucosal damage, respectively, were observed. Events with ≥ grade III renal impairment were not included in this study due to the small number of occurrences (2 events, 2.2%; *Table 3*).

Relationship between MTHFR polymorphisms and the 48-hour MTX blood concentration

The C677T gene polymorphism may increase the toxic response by decreasing MTX metabolism and maintaining high levels of 48-hour MTX blood concentrations (14,15). However, the present study found no significant correlation between the C677T gene polymorphism and 48-hour MTX blood concentrations (χ^2=2.298, P=0.317; *Table 4*).

The A1298C gene polymorphism and the 48-hour MTX blood concentrations were measured in 73 children with ALL, spanning a total of 281 HD-MTX sessions. No significant correlation was observed between the A1298C gene polymorphism and the 48-hour MTX blood concentrations (χ^2=0.564, P=0.754; *Table 5*).

Relationship between the 48-hour HD-MTX blood concentrations and common clinically relevant chemotherapeutic toxic reactions

A total of 513 sessions (56.1%) reached 48-hour MTX blood concentrations >0.2 μmol/L, while 402 sessions (43.9%) reached MTX concentrations ≤0.2 μmol/L. The relationship between the 48-hour blood concentration of MTX and the occurrence of grade II–IV toxic reactions was analyzed using the Chi-square test. A high 48-hour blood concentration of MTX significantly increased the incidence

Table 3 Common toxic reactions after chemotherapy with high-dose methotrexate in children with acute lymphoblastic leukemia

Toxic reactions	Grade II	Grade III	Grade IV	Total
Leukopenia	289	290	48	627
Neutropenia	188	200	93	481
Decreased haemoglobin	419	153	22	594
Thrombocytopenia	25	9	2	36
Liver function impairment	94			
Gastrointestinal reaction	67	4	0	71
Mucosal damage	122	11	0	133

Table 4 Correlation between MTHFR C677T gene polymorphism and 48-hour MTX blood concentration

Genotype	48-h MTX blood concentration (μmol/L)		χ^2	P value
	>0.2	≤0.2		
C677T			2.298	0.317
CC wild type	277	208		
CT+TT mutant type	236	194	0.460	0.498
CT heterozygous mutation	197	152		
TT homozygous mutation	39	42		

MTX, methotrexate; CC, wild-type; CT, heterozygous mutant; TT, homozygous mutant.

Table 5 Correlation between MTHFR A1298C gene polymorphism and 48-hour MTX blood concentration

Genotype	48-h MTX blood concentration (μmol/L)		χ^2	P value
	>0.2	≤0.2		
A1298C			0.564	0.754
AA wild type	43	118		
AC+CC mutant type	28	89	0.275	0.600
AC heterozygous mutation	22	74		
CC homozygous mutation	6	15		

MTX, methotrexate; AA, wild-type; AC, heterozygous mutant; CC, homozygous mutant.

of leukopenia and hemoglobinopenia (P<0.05), but had no significant effect on the incidence of neutropenia, thrombocytopenia, hepatic impairment, gastrointestinal reactions, nor mucosal damage (P>0.05). According to unconditional logistic regression model analysis, the risk of leukopenia and hemoglobinopenia were 1.749 (OR =1.749, 95% CI: 1.315–2.326, P<0.05) and 1.435 times (OR =1.435, 95% CI: 1.088–1.891, P<0.05) higher in patients with high 48-hour MTX blood levels than in those with low blood levels, respectively (*Table 6*).

Relationship between MTHFR gene polymorphisms and common clinically relevant chemotherapeutic toxic reactions to HD-MTX

Relationship between *MTHFR* C677T genotype and HD-MTX-related common toxic responses

The incidence of hemoglobinopenia, thrombocytopenia, hepatic impairment, and toxic reactions to mucosal damage were significantly different among the individual *MTHFR* C677T genotypes in children with ALL (*Tables 6*,*7*, P<0.05);

Table 6 Relationship between 48-h MTX blood concentrations and common clinically relevant grade II or higher chemotherapeutic toxic reactions

Toxic reactions (grades II-IV)	48-h MTX blood concentration (μmol/L)		χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
	>0.2	≤0.2				
Leukopenia	378	246	14.919	0.000	1.749	1.315–2.326
Neutropenia	276	204	0.581	0.446	1.108	0.851–1.442
Decreased hemoglobin	351	240	6.574	0.010	1.435	1.088–1.891
Thrombocytopenia	23	13	0.873	0.352	1.390	0.695–2.780
Liver function impairment	112	81	1.590	0.208	0.802	0.569–1.131
Gastrointestinal reaction	33	38	2.843	0.094	0.660	0.406–1.073
Mucosal damage	76	57	0.080	0.777	1.055	0.728–1.529

MTX, methotrexate.

Table 7 Relationship between *MTHFR* C677T gene polymorphisms and common high dose MTX-related grade II or higher toxic reactions

Toxic reactions	Times	*MTHFR* genotype			χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
		CC	CT	TT				
		CC	CT+TT					
Leukopenia	627	328	244	55	0.308	0.857		
		328	199		0.127	0.721	1.053	0.793–1.397
Neutropenia	481	264	180	37	2.967	0.227		
		264	217		1.955	0.162	0.830	0.639–1.078
Decreased hemoglobin	594	327	225	42	8.720	0.013		
		327	267		3.808	0.051	0.761	0.578–1.001
Thrombocytopenia	36	15	11	10	16.308	0.000		
		15	21		1.833	0.179	1.589	0.808–3.124
Liver function impairment	193	85	90	18	9.555	0.008		
		85	108		8.554	0.004	1.656	1.179–2.324
Gastrointestinal reaction	71	34	30	7	0.842	0.656		
		34	37		0.842	0.360	1.254	0.772–2.037
Mucosal damage	133	59	55	19	7.921	0.019		
		59	74		4.779	0.030	1.508	1.042–2.183

MTX, methotrexate; CC, wild-type; CT, heterozygous mutant; TT, homozygous mutant.

however, no significant differences in leukocytopenia, neutropenia, nor gastrointestinal reactions were observed (P>0.05). Furthermore, unconditional logistic regression model analysis revealed that the risk of hepatic impairment and mucosal damage were 1.656 (OR =1.656, 95% CI: 1.179–2.324, P<0.05) and 1.508 times (OR =1.508, 95% CI: 1.042–2.183, P<0.05) higher in patients with the CT+TT mutant than in those with the CC wild-type, respectively.

Relationship between each genotype of *MTHFR* A1298C and common HD-MTX-related toxic responses

The *MTHFR* A1298C genotypes differed significantly in

Table 8 The relationship between *MTHFR* A1298C gene polymorphisms and common high dose MTX-related grade II or higher toxic reactions

Toxic reactions	Times	*MTHFR* genotype			χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
		AA	AC	CC				
		AA	AC+CC					
Leukopenia	157	89	54	14	0.618	0.734		
		89	68		0.441	0.507	1.179	0.725–1.919
Neutropenia	117	74	34	9	2.093	0.351		
		74	43		1.980	0.160	0.704	0.431–1.149
Decreased hemoglobin	170	92	63	15	3.372	0.185		
		92	78		3.371	0.067	1.601	0.967–2.651
Thrombocytopenia	10	5	5	0	1.792	0.408		
		5	5		0.301	0.585	1.422	0.402–5.031
Liver function impairment	50	27	12	11	8.537	0.014		
		27	23		0.123	0.726	1.122	0.589–2.140
Gastrointestinal reaction	19	10	4	5	10.022	0.007		
		10	9		0.242	0.624	1.263	0.497–3.213
Mucosal damage	54	31	15	8	4.969	0.083		
		31	23		0.010	0.921	1.031	0.566–1.879

MTX, methotrexate; AA, wild-type; AC, heterozygous mutant; CC, homozygous mutant.

the incidence of hepatic impairment and gastrointestinal reactions (*Table 8*, P<0.05), but not in the incidence of bone marrow suppression and mucosal damage (P>0.05). Unconditional logistic regression analysis revealed that there was no increased risk of hepatic impairment nor gastrointestinal reactions in patients with the AC+CC mutant phenotype compared to those with the AA wild type.

Relationship between the different risk levels and HD-MTX-related toxic side effects

Children with ALL at different risk levels were administered HD-MTX in consolidation chemotherapy regimens at varying doses and in combination with chemotherapeutic agents that were not consistent with the SCCLG-ALL-2016 treatment regimen, leading to differences in toxic responses. As only 73 participants (281 MTX treatment sessions in total) were tested for the A1298C gene polymorphism, this study could only stratify the C677T gene polymorphism according to different risk levels of ALL rather than statistical analysis due to the limited data.

Low-risk group

A significant difference in the incidence of liver function damage and gastrointestinal reactions was observed among the *MTHFR* C677T genotypes in the low-risk group (*Table 9*, P<0.05), however, no significant differences were observed in bone marrow suppression nor mucosal damage (P>0.05). Furthermore, unconditional logistic regression model analysis revealed an elevated risk of hepatic impairment (OR =6.067, 95% CI: 1.183–31.102, P<0.05) and a lower risk of neutropenia (OR =0.498, 95% CI: 0.251–0.989, P<0.05) in patients with the CT+TT mutation compared to those with the CC wild type.

Intermediate-risk group

There was a significant difference in the incidences of hemoglobin and liver function impairment among the *MTHFR* C677T genotypes in the intermediate-risk group (*Table 10*, P<0.05), but no significant difference was observed in leukopenia, neutropenia, thrombocytopenia, gastrointestinal reactions, nor mucosal damage (P>0.05). Unconditional logistic regression model analysis further

Table 9 Relationship between *MTHFR* C677T gene polymorphism and common high dose MTX-related grade II or higher toxic reactions in the low-risk group

Toxic reactions	Times	*MTHFR* genotype			χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
		CC	CT	TT				
		CC	CT+TT					
Leukopenia	69	39	26	4	0.733	0.693		
		39	30		0.686	0.408	1.331	0.676–2.623
Neutropenia	72	49	20	3	4.056	0.132		
		49	23		4.008	0.047	0.498	0.251–0.989
Decreased hemoglobin	63	42	20	1	4.478	0.107		
		42	21		2.121	0.147	0.600	0.301–1.196
*Thrombocytopenia	2	0	2	0				
		0	2					
Liver function impairment	9	2	5	2	6.127	0.047		
		2	7		5.703	0.031	6.067	1.183–31.102
Gastrointestinal reaction	6	4	0	2	10.626	0.005		
		4	2		0.107	0.745	0.750	0.133–4.239
Mucosal damage	10	4	5	1	1.895	0.388		
		4	6		1.850	0.185	2.430	0.653–9.036

*, statistical analysis was not performed owing to limited positive data. MTX, methotrexate; CC, wild-type; CT, heterozygous mutant; TT, homozygous mutant.

revealed that there was no increased risk of toxic reactions in patients with the CT+TT mutant phenotype compared to those with the CC wild type.

High-risk group

A significant difference in the incidences of thrombocytopenia and mucosal damage was observed among the *MTHFR* C677T genotypes in the high-risk group (*Table 11*, P<0.05), while no significant differences were observed for leukopenia, neutropenia, hemoglobinopenia, hepatic impairment, nor gastrointestinal reactions (P>0.05). Moreover, unconditional logistic regression models revealed that the risk of mucosal damage in patients with CT+TT mutant was 1.906 times higher than in those with the CC wild type (OR =1.906, 95% CI: 1.033–3.518, P<0.05). However, an increased risk of thrombocytopenia was not observed (OR =0.855, 95% CI: 0.261–2.804, P>0.05).

Discussion

MTX is a commonly used drug in the treatment of many malignancies, including paediatric ALL. However, the use of MTX is often limited by drug-related toxicities such as bone marrow suppression, abnormal liver and kidney function, skin mucosal damage, and gastrointestinal reactions. Moreover, children can experience serious adverse reactions, even life-threatening reactions, even at doses which do not exceed the blood concentration limit. The forced reduction of drug dose during the subsequent MTX course is speculated to increase the risk of tumour recurrence. Therefore, it is necessary to investigate the mechanism of MTX-related toxic reactions to identify the susceptibility factors and improve patient outcomes. Recent studies have suggested that functional variants of the *MTHFR* gene may affect the efficacy and toxicity related to MTX (10-13). Indeed, the relationship between MTX blood concentration, polymorphisms of *MTHFR* C677T and A1298C gene loci, and the toxic response to HD-MTX warrants further clarification.

Some studies have suggested a correlation between MTX blood concentrations and toxic effects (16), and that the former may be used as an objective biomarker of MTX-

Table 10 Relationship between *MTHFR* C677T gene polymorphisms and common high dose MTX-related grade II or higher toxic reactions in the intermediate-risk group

Toxic reactions	Times	*MTHFR* genotype			χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
		CC	CT	TT				
		CC	CT+TT					
Leukopenia	454	237	180	37	4.863	0.088		
		237	217		2.793	0.095	0.718	0.486–1.060
Neutropenia	372	195	148	29	3.678	0.159		
		195	177		1.760	0.185	0.796	0.568–1.115
Decreased hemoglobin	382	202	153	27	7.697	0.021		
		202	180		2.828	0.093	0.747	0.531–1.050
Thrombocytopenia	22	8	9	5	5.224	0.073		
		8	14		1.786	0.187	1.813	0.749–4.390
Liver function impairment	112	49	56	7	7.104	0.029		
		49	63		3.068	0.081	1.481	0.953–2.299
Gastrointestinal reaction	32	11	19	2	5.368	0.068		
		11	21		3.704	0.059	2.056	0.973–4.345
Mucosal damage	56	25	24	7	1.342	0.511		
		25	31		0.975	0.325	1.320	0.759–2.296

MTX, methotrexate; CC, wild-type; CT, heterozygous mutant; TT, homozygous mutant.

related toxicity. Moreover, the sustained maintenance of high plasma MTX concentrations has been associated with the development of toxic drug reactions (17,18). Aumente and Avivi *et al.* proposed a 48-hour MTX blood concentration of 0.2 μmol/L as a clinical threshold for MTX toxic reactions (19,20), and this has been adopted in the current study. The chi-square test analysis suggested that the risk of leukopenia and hemoglobin reduction was significantly higher in patients with high MTX blood concentrations than in those with low MTX blood concentrations ($P<0.05$), and this was consistent with the findings of Liu *et al.* (21). Our data from South China provided additional support demonstrating that MTX blood concentrations can influence toxic effects. Notably, the active monitoring of MTX blood concentrations by clinicians can aid in predicting the degree of bone marrow suppression in children and facilitate clinical guidance for the timely transfusion of blood products, appropriate use of stimulating factors, and the prevention and control of infection.

In this study, no correlation was observed between the 48-hour blood concentration of MTX and the C677T and A1298C gene polymorphisms. Therefore, the relationship between polymorphisms at each gene locus and MTX drug concentrations could not be confirmed. Similarly, Chae *et al.* also reported no correlation between *MTHFR* genotypes and MTX blood concentrations (22). A meta-analysis that included 13 studies also suggested no correlation between MTX drug metabolism and C677T and A1298C gene polymorphisms, emphasizing that C677T and A1298C cannot be used as suitable predictors of MTX pharmacokinetics (16). However, Haase *et al.* observed significantly higher MTX blood concentrations in patients with 677CC wild type than in those with 677CT and 677TT mutations (23). This deviation could be attributed to the difference in the sample size between the present study and other previous studies.

A mutation in C677T, a common genetic polymorphism site of *MTHFR*, causes the substitution of cytosine at position 677 of *MTHFR* with thymine, and a corresponding amino acid sequence change from alanine to valine. The mutation significantly reduced the affinity of *MTHFR*

Table 11 Relationship between MTHFR C677T gene polymorphism and common high dose MTX-related grade II or higher toxic reactions in the high-risk group

Toxic reactions	Times	MTHFR genotype			χ^2	P value	Odds ratio (OR)	95% confidence interval (CI)
		CC	CT	TT				
		CC	CT+TT					
Leukopenia	104	52	38	14	4.012	0.134		
		52	52		2.259	0.134	1.586	0.868–2.899
Neutropenia	37	20	12	5	0.660	0.719		
		20	17		0.009	0.924	1.036	0.501–2.141
Decreased hemoglobin	149	83	52	14	0.556	0.757		
		83	66		0.328	0.567	0.795	0.363–1.744
Thrombocytopenia	12	7	0	5	17.338	0.000		
		7	5		0.067	0.796	0.855	0.261–2.804
Liver function impairment	72	34	29	9	3.202	0.202		
		34	38		2.632	0.106	1.676	0.896–3.135
Gastrointestinal reaction	33	19	11	3	0.136	0.934		
		19	14		0.131	0.718	0.869	0.405–1.864
Mucosal damage	67	30	26	11	6.652	0.036		
		30	37		4.298	0.039	1.906	1.033–3.518

MTX, methotrexate; CC, wild-type; CT, heterozygous mutant; TT, homozygous mutant.

for the cofactor flavin adenine dinucleotide, subsequently decreasing MTHFR stability, heat resistance, and enzymatic activity (24,25). Mutant states of 677CT and 677TT encode only 60% and 30%, respectively, of the MTHFR activity encoded by the wild type (677CC), which reduces the metabolism rate and increases MTX accumulation. Notably, MTX commonly causes liver function impairment. Although a few studies have suggested no correlation between C677T mutations and liver function impairment (26,27), a great number of other studies have indicated a strong association. In a meta-analysis by Zhu *et al.* that included 14 studies with a sample size of 1714 children, the C677T mutant gene was significantly associated with hepatotoxicity (28). Additionally, several reports have confirmed a significantly higher risk of hepatic impairment in patients with mutant genotypes (29-32). El-Khodary *et al.* also demonstrated significantly higher serum glutaminase levels in children with ALL who have CT and TT mutation compared to those with wild-type CC (33). In this study, we found significant differences in the incidence of hepatic impairment among *MTHFR* C677T genotypes. Additionally, the risk of hepatic impairment was significantly higher in patients with the CT+TT mutation than in those with the CC wild-type. Another stratified analysis reported the same conclusion in the low-risk group. The C677T mutation has been shown to cause a rapid increase in cysteine levels *in vivo* after MTX chemotherapy, which could be a potential molecular mechanism leading to liver function impairment (34). Regarding myelosuppression, Giletti *et al.* concluded that the TT mutant phenotype significantly reduced the hematologic toxic response to MTX (35). However, some studies reported no correlation between the MTHFR CT+TT mutant phenotype and hematologic toxicity (36,37). In the present study, we found that the risk of neutropenia was lower in patients with the CT+TT mutation in the low-risk group than in those with the CC wild-type. However, no correlation was observed in the intermediate-risk and high-risk groups. Several studies have demonstrated the absence of a correlation between C677T gene polymorphism and mucosal damage (16,38). However, a study that included 109 children with ALL found that the risk of mucositis in children with the CC

wild-type was 3 times higher than those with the CT+TT mutation. Thus, mutant genes are considered to induce a protective effect against mucosal damage, whereas wild-type genes are more likely to cause mucosal damage (39). In contrast, some studies also reported that C677T mutant genes are strongly associated with grade III or higher mucosal toxicity, thereby causing oral mucositis (28,29,40). In the present study, patients with the CT+TT mutant phenotype were more prone to mucosal damage in the high-risk group, but no correlation was observed in the low- and intermediate-risk groups. Furthermore, MTX can be secreted from saliva, leading to increased direct mucosal toxicity, altered glutathione metabolism, and altered gastrointestinal microbiota. Mucositis may be induced via different inflammatory responses, such as pro-inflammatory cytokines and folate metabolism pathway genes (18). Gong *et al.* also confirmed that the CT+TT mutant genes can increase the mucosal toxicity of MTX (41). Thus, the *MTHFR* C677T gene polymorphism increases the risk of liver toxicity and mucosal damage in children with ALL treated with HD-MTX regimens. Therefore, changes in liver function, and skin and oral mucosa should be closely monitored during HD-MTX chemotherapy. In children with the C677T mutation, the dose of MTX treatment can be personalised accordingly. Additionally, intensive adjuvant supportive therapy, such as hydration and alkalinization, intensive liver protection, and intensive CF mouthwash, should be appropriately administered during chemotherapy.

Another common gene polymorphism locus for *MTHFR* is A1298C, which results in the conversion of glutamate to alanine at codon 429. MTHFR activity encoded by mutant 1298CC is 60% of that encoded by wild-type 1298AA. However, only 30% of MTHFR enzyme activity remains when two different alleles of MTHFR are present, 677T and 1298C (16,30,42,43). Moreover, A1298C polymorphism was not significantly correlated with the occurrence of MTX-induced side effects (28,37). However, some studies have found that the AC+CC mutant phenotype leads to an increased risk of myelosuppression and liver function impairment (44,45). In contrast, another study reported a significantly lower risk of neutropenia and hepatotoxicity in children with the CC mutation. The *MTHFR* A1298C mutation reduces enzyme activity, thereby increasing the substrates available for thymidine synthesis and consequently increasing DNA synthesis and reducing the incidence of toxic reactions to MTX (21,46,47). In the present study, significant differences in the incidence of liver function impairment and gastrointestinal reactions were found among genotypes. However, an increased risk of hepatic impairment and gastrointestinal reactions in children with the AC+CC mutant was not observed in the unconditional logistic regression model, which could be attributed to insufficient sample size. Therefore, the relationship between the A1298C polymorphism and the toxic side effects of MTX drugs warrants further investigation.

Although the relationship between *MTHFR* C677T and A1298C gene polymorphisms and MTX toxic response has been extensively studied, definitive conclusions and uniform consensus remain unavailable. The diversity of the findings could be attributed to the differences in treatment regimens used, varying doses of MTX, different treatment experience and levels at different research institutions, smaller study populations, ethnic heterogeneity and failure to homogenize the items and time points for evaluating toxic responses. Additionally, there are some uncontrollable confounding factors. For example, there are many other enzymes or transporter proteins such as Folypolyglutamate synthase (FPGS) (12), gamma-glutamyl hydrolase (GGH) (48), solute carrier organic anion transporter 1B1(SLCO1B1) (49), and ATP-binding cassette B1(ABCB1) (50) that have an impact on MTX pharmacokinetic variability in addition to MTHFR. During HD-MTX treatment, other drugs, such as mercaptopurine, or prophylactic agents against fungi may also be used. These factors may act together and influence the extent of toxic reactions experienced by the pediatric patient. The present study, as a multicentre retrospective study with large sample size, did have the above limitations. Therefore, in subsequent studies, the treatment process should be further standardized across hospitals and the study population size should be expanded to homogenize the assessment items and time. Furthermore, other genes and polymorphic loci related to MTX metabolism should be included. Additionally, the molecular biological mechanisms related to MTX metabolism should be explored in conjunction with basic research to provide a more reliable basis for developing personalised treatment regimens of MTX in the clinical setting.

Conclusions

Despite a lack of consensus on the relationship between MTX blood levels, polymorphisms at the *MTHFR* C677T and A1298C loci, and the toxic response to HD-MTX, this study provided additional data related to the MTX treatment of children with ALL from southern China.

These findings encourage clinicians to actively improve *MTHFR* genotype testing in children with ALL and recommend individualized treatment regimens during HD-MTX chemotherapy based on the genotype results. Thus, this study aids in predicting, preventing, and reducing the occurrence of serious MTX-related toxic reactions.

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Footnote

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