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Effect of the Polymorphism of Folylpolyglutamate Synthetase on Treatment of High-Dose Methotrexate in Pediatric Patients with Acute Lymphocytic Leukemia

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to investigate the association of the polymorphism of folylpolyglutamate synthetase (FPGS) with the dynamic plasma concentration of methotrexate (MTX) in pediatric patients with acute lymphocytic leukemia (ALL), as well as the prognosis.

Material/Methods: 57 ALL patients and 31 age and sex-matched children (control) were included in this study. Polymerase chain reaction-restriction fragment length polymorphism was performed for the analysis of the genotype of FPGS rs1544105 and high-performance liquid chromatography for measurement of MTX plasma concentration after 24-h and 44-h treatment. Overall survival was analyzed by Kaplan-Meier method.

Results: No differences were observed between patients and controls regarding the distribution frequency of genotype and alleles of rs1544105. Patients carrying AA genotype had a significantly higher plasma concentration of MTX after 24 h than those carrying GG or GA ($P<0.05$) and no differences were found after 44 h. Kaplan-Meier survival analysis showed a longer median survival time in patients with AA than other genotypes with significant difference in overall survival.

Conclusions: Polymorphism of FPGS rs1544105 might be used as an effective approach for prediction of the treatment outcome of MTX.

MeSH Keywords: gamma-Glutamyl Hydrolase • Polymorphism, Single Nucleotide • Precursor Cell Lymphoblastic Leukemia-Lymphoma

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Background

Acute lymphocytic leukemia (ALL) is one of the most common hematologic malignancies in children. High-dose methotrexate (HD-MTX) has been shown to play an important role in the prophylaxis and treatment of extramedullary leukemia [1]. Different treatment efficiency and adverse effects were observed in different individuals even with the same dose of MTX, suggesting the existence of differences in the MTX metabolism *in vivo* [2]. As a catalase, folylpolyglutamate synthetase (FPGS) plays a critical role in the metabolism of MTX, and its deficiency can cause a dramatic reduction of intracellular MTX polyglutamate (MTXPG) level, leading to MTX resistance in leukemia cells [3,4], which are further supported by the increased sensitivity of several glioma cells to MTX after overexpression of FPGS [5]. In addition, a correlation of FPGS expression with intracellular MTXPG level and treatment outcome has been observed in pediatric patients with ALL [6,7]. FPGS rs1544105, resulting from C to T, has been identified as a functional polymorphism affecting FPGS activity in pediatric patients with ALL [8]. Considering the important role of FPGS playing in MTX metabolism, whether FPGS's function polymorphism rs1544105 affects the treatment of MTX in pediatric patients with ALL remains poorly understood. In this study, we aimed to analyze the genotype of rs1544105 in pediatric ALL patients and to conduct a preliminary investigation of the relationship between rs1544105 genotype with MTX plasma concentration or ALL prognosis.

Material and Methods

Patients

From July 2012 to October 2014, 57 pediatric patients with newly diagnosed ALL were included in this study. The diagnosis of ALL was based on international guidelines [9] and further confirmed by bone marrow cytology. All patients achieved remission after treatment with VDLP (Vincristine, Daunorubicin, L-asparaginase, and Prednisone). High-dose methotrexate was used for the prophylaxis of lymphocytic leukemia. Those patients were excluded from this study, including those who left the hospital or died before the end of 1 course of treatment, had impaired function of liver or kidney before chemotherapy, or who received both chemotherapy and radiotherapy. Thirty-one age and sex-matched healthy children were served as a control group. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University. Informed consent was obtained from all participants.

Reagents

Methotrexate (batch no. HY21A) was purchased from Ruihui Pharmaceutical Co., Ltd. Reference substance for methotrexate (batch no. HWG00526) and levofloxacin (batch no. 130537-200301) were from the Chinese Institute for Drug Control.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Genomic DNA was extracted from patients or control followed by PCR-RFLP analysis of the distribution of rs1544105 genotype, as previously described [10]. Briefly, PCR amplification was performed with the following conditions: Initial denaturation (95°C for 10 min), 40 cycles of denaturation (95°C for 15 s), annealing (60°C for 1 min) and extension (72°C for 1 min), and final extension (72°C for 7 min). Sequences for PCR primers were: Forward: 5'-CCCAGAGTCCTTATTCTTAGCC-3' and Reverse: 5'-GTGCCTCCTTCACACACAG-3'. After completion of amplification, restriction enzyme HpyCH4IV was added into the PCR product and incubated at 37°C for 4 h, followed by 2.5% agarose gel electrophoresis for analysis of RFLP.

Measurement of MTX concentration

Peripheral venous blood was drawn from patients at 24 h or 44 h after MTX treatment, followed by measurement of MTX concentration by HPLC, as described previously [11]. The conditions used for chromatographic analysis were as follows: stationary phase [ZORBAX XDB-C18 (4.6×250 mm, 5 μm, Agilent, USA)], guard column [XDB-C18 (4.6×12.5 mm, 5 μm)], mobile phase [methyl cyanide /H_2O: 30/70 (V/V), rate: 1 ml/min, column temperature: 30°C]. The detection wavelength was 225 nm.

Statistical analysis

SPSS 19.0 software was used for statistical analysis. Hardy-Weinberg equilibrium of genotype distribution as well as the difference of genotype in sex and risk group were evaluated by chi-square test. Comparison of MTX concentration within different genotypes was performed by one-way ANOVA or Kruskal-Wallis test. Overall survival was assessed by Kaplan-Meier method. $P<0.05$ was considered to be a statistically significant difference.

Results

Patients' characteristics

This study included 57 pediatric ALL patients and 31 healthy children. No significant differences were observed in these 2 groups in sex or age. Detailed information is listed in Table 1.

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Table 1. Clinical characteristics of patients with ALL and the control group.

Parameters	ALL group	The control group
Age(years)	5.9±4.3	3.7±1.6
Gender(n)		
Male	31	18
Female	26	13
ALL (n)		
T-Lineage	11	
B-Lineage	46	
Risk (n)		
Standard	21	
Intermediate	27	
High	9	
WBC (10^9 L)		
<50	52	
≥50	5	

Polymorphism of FPGS rs1544105

Polymorphism of rs1544105 was evaluated using the PCR-RFLP approach. After PCR amplification of FPGS, restricted enzyme HpyCH4IV was used to detect the mutation of rs1544105. After HpyCH4IV treatment on PCR product, 3 genotypes were detected based on the length after electrophoresis: homozygotes AA (298 bp), heterozygotes GA (298 bp, 168 bp, and 130 bp), and homozygotes GG (168 bp and 130 bp) (Figure 1).

Genotype and alleles of FPGF rs1544105

The genotype distribution of FPGS rs1544105 in ALL patients and healthy controls was in accordance with Hardy-Weinberg equilibrium (P>0.05). The distribution frequency of alleles was similar in these 2 groups. Compared to allele A, odds ratio (OR) of allele G was 0.97, with no significant difference (Table 2).

Chromatographic analysis of MTX

Chromatomaps for blank plasma (Figure 2A), MTX plasma standard (Figure 2B), and patient plasma (Figure 2C) were obtained through HPLC analysis (Figure 2). Chromatographic peak of MTX could be separated completely without obvious interference of endogenous impurity peak. The retention time for MTX and levofloxacin (internal standard) was 4.92 min and 9.42 min, respectively. A significant linear relation between MTX concentration and the ratio of MTX to internal standard peak area (Ai/As) was observed with regression equation being C=6.1328Ai/As – 0.0195 (r=0.9999) when MTX concentration was in 0.03–5.00 μg/ml, which was obtained from the standard curve under the current condition in this study. The lowest detected concentration was 0.03 μg/ml (RS, N=3). The recovery rate was 100.317±2.401%. Intra- and inter-batch errors

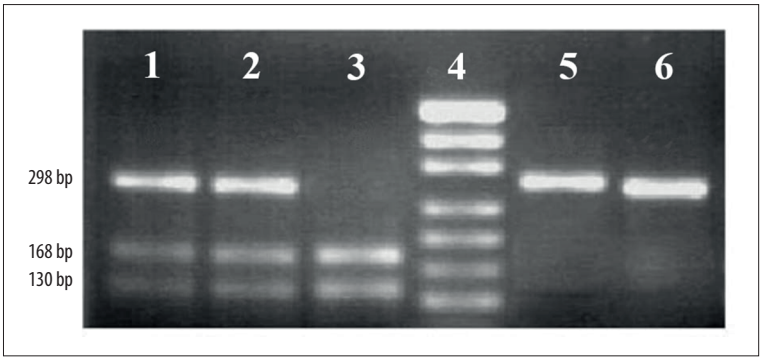

Figure 1. Electrophoresis of PCR-RFLP product. Genomic DNA was isolated from patients or controls, followed by analysis of the polymorphism of FPGS rs1544105 by PCR-RFLP. Lane 1 and 2: GA, Lane 3: GG, Lane 4: Marker, Lane 5 and 6: AA.

Table 2. Distribution of FPGS rs1544105 polymorphism in ALL and control.

	Genotype (%)			Gene frequency		OR	95%CI
	GG	GA	AA	G	A		
ALL (n=57)	11 (19.30%)	21 (36.84%)	25 (43.86%)	43 (37.72%)	71 (62.28%)	0.965	0.764–1.219
Control (n=31)	4 (12.90%)	14 (45.16%)	13 (41.94%)	22 (35.48%)	40 (64.52%)	1	
P		0.654[a]			0.769[b]		

Chi-square test: [a] F=0.848, [b] F=0.086. OR – odds ratio.

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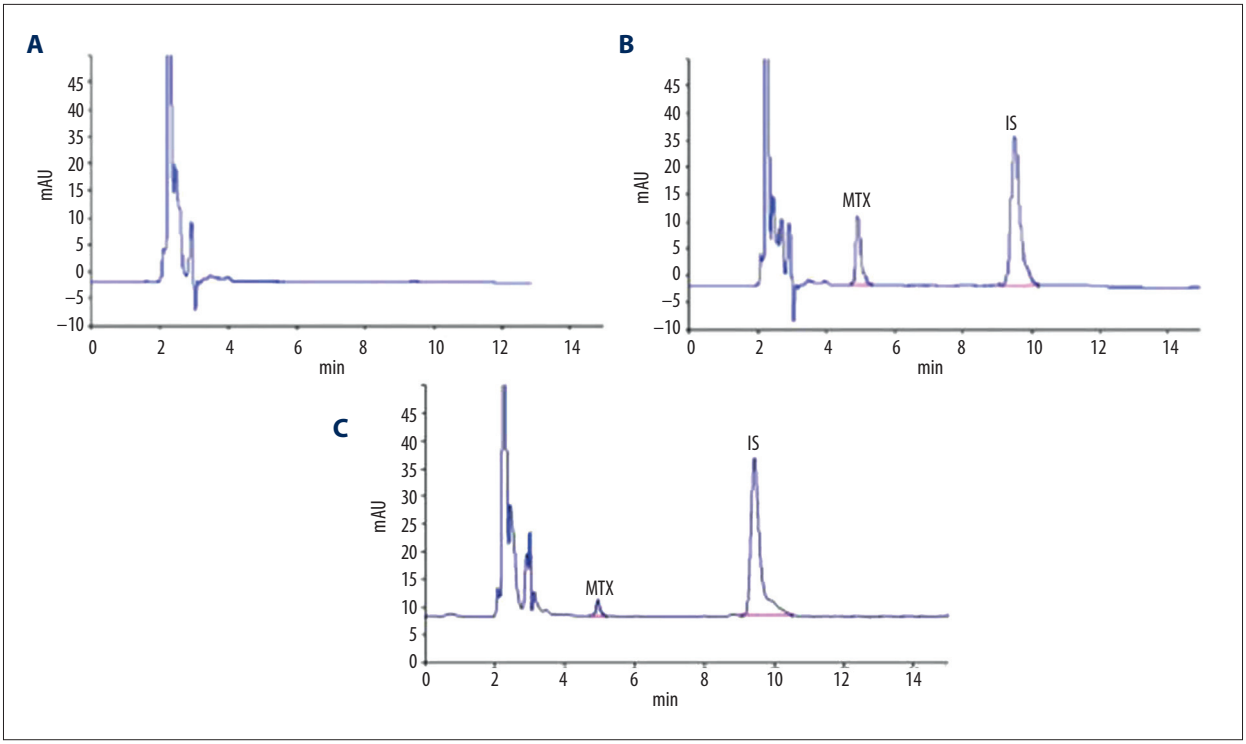

Figure 2. HPLC analysis of the plasma concentration of MTX. Through using blank plasma (**A**) and MTX plasma standard (**B**), plasma concentration of MTX (**C**) after administration was measured by HPLC.

Table 3. Relationship between FPGS genotype and concentration of MTX.

Time after MTX infusion	FPGS genotype	C/D (mmol/L per g/m²)	F	P
24 hour	AA	15.30±2.98	4.125	0.023[a]
	AG	14.14±3.19		
	GG	12.19±2.59		
44 hour	AA	0.11±0.04	3.065	0.055[b]
	AG	0.11±0.05		
	GG	0.08±0.03		

[a] P – One-way ANOVA analysis; [b] P – Kruskal-Wallis test.

as measured by precision test were 5.88% and 6.50%, respectively. MTX plasma samples were stable regardless of the storage condition, such as room temperature, frozen preservation, or freeze-thaw condition.

Relationship between polymorphism of FPGS rs1544105 and MTX concentration

In this study, we used dose-corrected MTX concentration per body surface area as an indicator to investigate the effect of polymorphism on MTX concentration. Our results showed that MTX C/D value (at 24 h) was significantly higher in ALL patients with AA genotype of rs1544105 than in patients with other genotypes (P<0.05) (Table 3). However, no difference in MTX C/D value was observed within different genotypes at 44 h (Table 3).

Association of rs1544105 polymorphism with prognosis

The follow-up period in this study was from July 2012 to October 2014. Kaplan-Meier method was used for analysis of the association of rs1544105 polymorphism with survival time. Our results revealed a significant difference of overall survival between patients with AA genotype and GA + GG genotype (P<0.05) (Table 4). After adjustment of white blood cell count, sex, age, and immune typing by Cox multivariate

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Table 4. Overall survival analysis in ALL patients with different FPGS genotypes.

Group	Median survival time (w)		95% Confidence Interval (w)		F	P
	Estimate	Std Error	Lower bound	Upper bound		
Overall	37.400	6.912	23.853	50.947		
AA	52.000	12.079	28.326	75.674		
AG+GG	35.000	4.011	27.139	42.861	4.352	0.037[a]

[a] P – Log rank test.

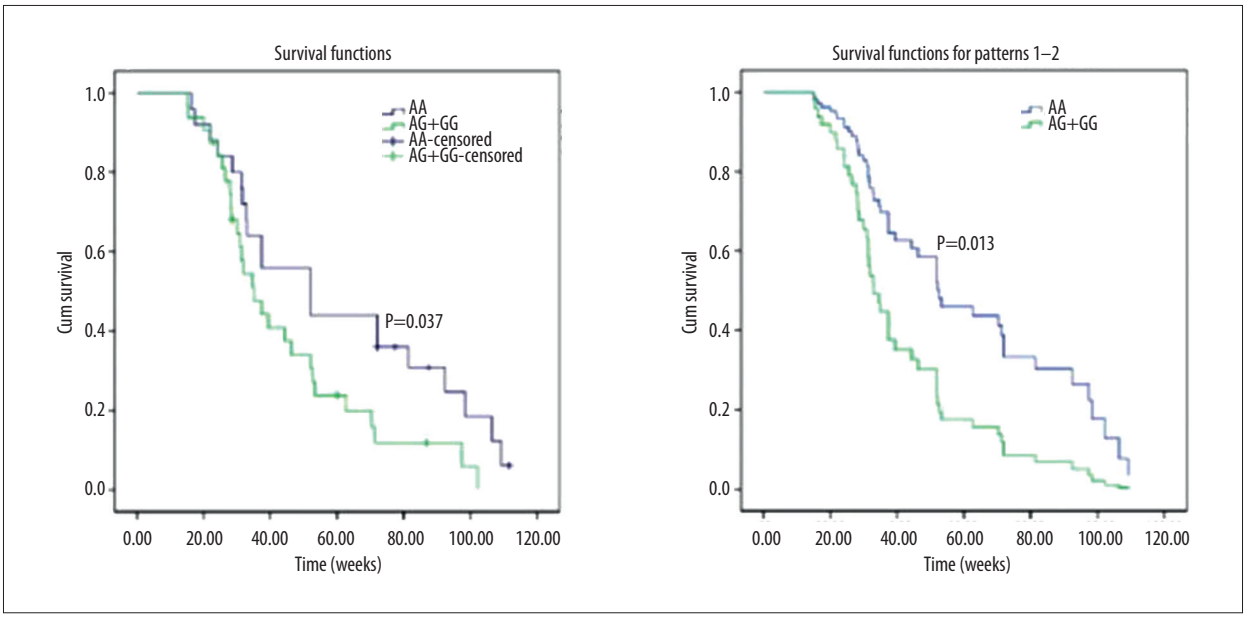

Figure 3. Analysis of overall survival and Cox regression in ALL patients with different genotypes. Overall survival in patients treated with MTX was analyzed by Kaplan-Meier method.

analysis, FPGS rs1544105 alleles were demonstrated to be an indicator for poor prognosis (log rank P=0.013, hazard ratio: 0.447, 95%CI: 0.237–0.842) (Figure 3).

Discussion

As an anti-tumor drug in the chemotherapy of pediatric ALL patients, methotrexate (MTX) plays an important role in the consolidation and maintenance treatment. In the clinic, plasma concentration of MTX and release of calcium folinate are monitored dynamically to reduce the occurrence of chemotherapy-induced adverse effects. A previous study demonstrated the existence of individual differences in the pharmacokinetics, effectiveness, and toxicity of MTX, suggesting there might be some potential gene targets affecting the pharmacokinetics and pharmacodynamics of MTX [12]. FPGS is an important enzyme in MTX metabolism, and catalyzed poly glutamic acid is the core of folate antagonists-associated cytotoxic therapy [13,14]. Therefore, investigating the effect of FPGS polymorphism on drug treatment in ALL patients would have some important guiding significance [15].

Through analysis of the polymorphism of FPGS rs1544105, we found that the genotype distribution in ALL patients and healthy controls were in accordance with Hardy-Weinberg equilibrium. The distribution frequencies of GG, GA, and AA genotype in healthy controls were close to HAPMap-HCB (Han Chinese in Beijing, China), which was 11.6%, 39.5%, and 48.8%, respectively [16]. In this study we found the distribution frequency of alleles in ALL patients was close to that in controls, indicating no significant correlation of FPGS polymorphism with the pathogenesis of ALL, consistent with a previous study [17].

According to recommendations for ALL diagnosis and treatment, MTX doses for patients with low, medium, or high risk are different. To exclude the interference of different doses, dose-standardized MTX plasma concentration was used as an analysis indicator to monitor the dynamical concentration of MTX. Our results showed a significant difference in MTX

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concentration among patients with different genotypes at 24 h, showing a higher concentration for AA genotype than GA and GC (P<0.05). However, no difference was observed at 44 h. There are few reports on the relationship between FPGS rs1544105 polymorphism and the sensitivity and toxicity of MTX-associated chemotherapy, with contradicting results. Liu et al. [18] and Panetta et al. [19] demonstrated that FPGS activity was higher in individuals with GG than other genotypes with higher MTX plasma concentration as well as increased sensitivity to MTX. Sharma et al [17]showed allele G might reduce the MTX treatment efficiency. In our study a difference of MTX concentration was found within different genotypes at 24 h but not 44 h. There may be several reasons to explain this. First, MTX metabolism is regulated by multiple genes, such as methylene tetrahydrofolate reductase, and γ-glutamyltransferase [20,21]. The exact role of each gene in MTX metabolism is not clear, and the interaction of each gene with FPGS makes it difficult to interpret the results we obtained. Second, a single time point was chosen in this study to reflect the system exposure extent of the drug, which might have had a large influence on the result precision when the plasma concentration was lower, which might be why no difference was found at 44 h. Third, the Kruskal-Wallis test was performed on the samples at 44 h because they were not in normal distribution (P=0.032) and the P value was equal to 0.055. Therefore, a large sample-size study was required to confirm the finding.

In this study we found higher efficacy of chemotherapy in patients with AA genotype than in those with GA and GG, further supported by the overall survival. The median survival time of patients with AA was significantly longer than in patients with other genotypes, with a significant difference of overall survival. Regarding the studies on MTX resistance, Stark et al. [22] found impaired activity of FPGS in patients with MTX resistance. Further study in these patients demonstrated a defect in FPGS mRNA splicing, leading to premature termination and subsequent FPGS dysfunction and drug resistance. However, the exact mechanism by which this polymorphism affects FPGS function is not clear and requires further investigation.

Conclusions

Our study demonstrated a correlation of the polymorphism of FPGS rs1544105 with MTX treatment efficacy, as well as with overall survival, suggesting it could be used as an effective approach in the prediction of MTX treatment efficacy.

Conflict of interests

The authors have declared that no competing interest exists.

References:

1. Mantadakis E, Cole PD, Kamen BA: High-dose methotrexate in acute lymphoblastic leukemia: Where is the evidence for its continued use? Pharmacotherapy, 2005; 25: 748–55
2. Huang L, Tissing WJ, de Jonge R et al: Polymorphisms in folate-related genes: Association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. Leukemia, 2008; 22: 1798–800
3. Gangjee A, Jain HD, Kurup S: Recent advances in classical and non-classical antifolates as antitumor and antiopportunistic infection agents: Part I. Anticancer Agents Med Chem, 2007; 7: 524–42
4. Leclerc GJ, Mou C, Leclerc GM et al: Histone deacetylase inhibitors induce FPGS mRNA expression and intracellular accumulation of long-chain methotrexate polyglutamates in childhood acute lymphoblastic leukemia: implications for combination therapy. Leukemia, 2010; 24: 552–62
5. Aghi M, Kramm CM, Breakefield XO: Folylpolyglutamyl synthetase gene transfer and glioma antifolate sensitivity in culture and *in vivo*. J Natl Cancer Inst, 1999; 91: 1233–41
6. Galpin AJ, Schuetz JD, Masson E et al: Differences in folylpolyglutamate synthetase and dihydrofolate reductase expression in human B-lineage versus T-lineage leukemic lymphoblasts: Mechanisms for lineage differences in methotrexate polyglutamylation and cytotoxicity. Mol Pharmacol, 1997; 52: 155–63
7. Rots MG, Willey JC, Jansen G et al: mRNA expression levels of methotrexate resistance-related proteins in childhood leukemia as determined by a standardized competitive template-based RT-PCR method. Leukemia, 2000; 14: 2166–75
8. Panetta JC, Sparreboom A, Pui CH et al: Modeling mechanisms of *in vivo* variability in methotrexate accumulation and folate pathway inhibition in acute lymphoblastic leukemia cells. PLoS Comput Biol, 2010; 6: e1001019
9. Alvarnas JC, Brown PA, Aoun P et al: Acute lymphoblastic leukemia. J Natl Compr Canc Netw, 2012; 10: 858–914
10. Sharma S, Das M, Kumar A et al: Purine biosynthetic pathway genes and methotrexate response in rheumatoid arthritis patients among north Indians. Pharmacogenet Genomics, 2009; 19: 823–28
11. Li YD, Li Y, Liang NS et al: A reversed-phase high performance liquid chromatography method for quantification of methotrexate in cancer patients serum. J Chromatogr B Analyt Technol Biomed Life Sci, 2015; 1002: 107–12
12. Lonnerholm G, Valsecchi MG, De Lorenzo P et al: Pharmacokinetics of high-dose methotrexate in infants treated for acute lymphoblastic leukemia. Pediatr Blood Cancer, 2009; 52: 596–601
13. Izbicka E, Diaz A, Streeper R et al: Distinct mechanistic activity profile of pralatrexate in comparison to other antifolates in *in vitro* and *in vivo* models of human cancers. Cancer Chemoth Pharm, 2009; 64: 993–99
14. Liani E, Rothem L, Bunni MA et al: Loss of folylpoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. Int J Cancer, 2003; 103: 587–99
15. Leclerc GJ, York TA, Hsieh-Kinser T, Barredo JC: Molecular basis for decreased folylpoly-gamma-glutamate synthetase expression in a methotrexate resistant CCRF-CEM mutant cell line. Leukemia Res, 2007; 31: 293–99
16. Wang SM, Sun LL, Zeng WX et al: Influence of genetic polymorphisms of FPGS, GGH, and MTHFR on serum methotrexate levels in Chinese children with acute lymphoblastic leukemia. Cancer Chemoth Pharm, 2014; 74: 283–89
17. Sharma S, Das M, Kumar A et al: Interaction of genes from influx-metabolism-efflux pathway and their influence on methotrexate efficacy in rheumatoid arthritis patients among Indians. Pharmacogenet Genom, 2008; 18: 1041–49
18. Liu SG, Li ZG, Cui L et al: Effects of methylenetetrahydrofolate reductase gene polymorphisms on toxicities during consolidation therapy in pediatric acute lymphoblastic leukemia in a Chinese population. Leukemia Lymphoma, 2011; 52: 1030–40

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19. Panetta JC, Sparreboom A, Pui CH et al: Modeling mechanisms of *in vivo* variability in methotrexate accumulation and folate pathway inhibition in acute lymphoblastic leukemia cells. PLoS Comput Biol, 2010; 6(12): e1001019
20. Nersting J, Schmiegelow K: Pharmacogenomics of methotrexate: Moving towards individualized therapy. Pharmacogenomics, 2009; 10: 1887–89
21. Kremer JM: Methotrexate pharmacogenomics. Ann Rheum Dis, 2006; 65: 1121–23
22. Stark M, Wichman C, Avivi I, Assaraf YG: Aberrant splicing of folylpolyglutamate synthetase as a novel mechanism of antifolate resistance in leukemia. Blood, 2009; 113: 4362–69

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