RESEARCH ARTICLE

Association Between Polymorphisms of Dihydrofolate Reductase and Gamma Glutamyl Hydrolase Genes and Toxicity of High Dose Methotrexate in Children with Acute Lymphoblastic Leukemia

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Abstract

Methotrexate (MTX) is an important drug for the treatment of childhood acute lymphoblastic leukemia (ALL). However, related toxicity occurs in many organs which may cause interruption of treatment, morbidity, and mortality. Single nucleotide polymorphisms (SNPs) of dihydrofolate reductase (DHFR) and gamma glutamyl hydrolase (GGH) are known to alter their enzymatic activity and thus affect the metabolism of MTX and influence the effectiveness. Therefore, we hypothesized that genetic variations of DHFR and GGH genes may influence the risk of toxicity after high dose MTX. The study population comprised of 105 children with ALL who were treated according to the modified St Jude Total XV protocol. The patients received 2.5 or 5 g/m^2 of MTX for 5 doses during the consolidation phase. Genotyping of DHFR 829C>T and GGH -401C>T was performed using a polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). The GGH-401CT and TT genotypes were associated with increased risk of leukopenia and thrombocytopenia after high dose MTX (OR 2.97, 95%CI; 1.24-7.13 and OR 4.02, 95%CI; 1.58-10.26). DHFR 829C>T was not associated with toxicity. In conclusion, the GGH -401CT and TT genotypes were found to increase the risk of severe leukopenia and thrombocytopenia after exposure to high dose MTX for childhood ALL therapy.

Keywords: Acute lymphoblastic leukemia - methotrexate - - toxicity - DHFR - GGH - polymorphisms

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Introduction

Methotrexate (MTX) is an antifolate drug that used in the treatment of children with acute lymphoblastic leukemia (ALL). After drug entry into the cell, its main mechanism is inhibition of dihydrofolate reductase (DHFR) enzyme. DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate required for the synthesis of thymidine and purines. Moreover, gamma glutamyl hydrolase (GGH) is a lysosomal peptidase that catalyses the removal of gamma-linked polyglutamates, converting a long-chain methotrexate polyglutamates (MTX-PGs) into a short-chain MTX-PGs and ultimately to MTX. This mechanism decreases therapeutic activity of MTX (Chabner et al., 1985; Gorlick et al., 1996; Rhee et al., 1998; Galivan et al., 1999; Genestier et al., 2000; Panetta et al., 2002; Fotoohi and Albertioni, 2008).

Individual polymorphisms in genes encoding for drug-metabolizing enzymes, transporters, and drug targets effect the toxicity from chemotherapeutic agents (Evans and Relling, 1999; Relling and Dervieux, 2001). DHFR 829C>T is a single nucleotide polymorphism (SNP) which identified within the 3'-UTR. The DHFR 829TT genotype increased mRNA and protein levels (Goto et al., 2001; Prasunkumar et al., 2006). GGH –401C>T polymorphism is the promoter polymorphism enhanced GGH expression that is associated with resistance to MTX in patients with ALL (Chave et al., 2003; Dervieux et al., 2004).

Our hypothesis was that the non-synonymous SNPs of DHFR andGGH may affect the side effects of MTX due to the alterations of expression and activity of these enzymes. Therefore, the aim of this study was to investigate whether DHFR829C>T and GGH-401C>T were associated with the adverse effects of high dose MTX given to children with ALL.

Materials and Methods

Patients

The subjects were children aged less than 15 years who were diagnosed with ALL at Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand between 2004 and 2010. Diagnosis of ALL was made by using morphological, cytochemical, and

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immunophenotyping methods. The treatment guideline was modified from the St Jude Total therapy XV (St Jude Children's Research Hospital, 2005). The patients received MTX 2.5 g/m^2/dose (low risk group) or 5 g/m^2/dose (standard and high risk groups) for 5 doses every 2 weeks during consolidation phase. We used 100 DNA samples from blood donors as a control group for the frequencies of DHFR and GGH polymorphisms.

Genotyping analysis of DHFR and GGH genes

The DHFR 829C>T polymorphism was detected using a polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). The primers for DHFR 829C>T (forward 5'- AGTTGTTCCCCCTCCCTCT -3', reverse 5'-GCTCAGTTGAAGGGTATGTGG -3') were designed by primer 3 software. The amplification protocol was composed of 2.5 μl 10 X PCR buffer, 0.75μl 50 mM $MgCl_2$, 2.0 μl 10 mM dNTPs,0.5 μl 10 μM of each of the forward and reverse primers, 0.2 μl 5 U/μl of Taq DNA polymerase enzyme and 16.55 μl sterile dH_2O was added to a final volume of 25 μL of reaction mixture. The PCR reaction consisted of pre denaturation step of 5 min at 95 °C followed by 30 denaturation cycles of 45 sec at 94 °C, annealing step of 45 sec at 60 ºC and extension at 72 ºC for 45 sec and post extension at 72 ºC for 10 min. The PCR product was digested by TaaI enzyme. The amplified PCR products (322 bp) and restriction products were electrophoresis on 2% agarose gel. The restriction fragments of DHFR 829C>T were presented as CC (202and120 bp), CT (322, 202 and 120 bp), and TT genotype (322 bp).

GGH -401C>T polymorphism was detected using a PCR-RFLP method. The primers for GGH -401C>T were forward 5'- CGCTGCCTGGTTACCAAA-3'and reverse 5'- CGTCCTTCCCTTTCAACTGT -3'. The 0.2 μl 5U/μl of TaqDNA polymerase enzyme and 16.55 μl sterile dH2O were added to a final volume of 25μl. The PCR reaction was performed using pre denaturation step of 5 min at 95 °C followed by 35 denaturation cycles of 1 min at 95 °C, annealing of 1 min at 57.8 ºC and extension at 72 ºC for 1 min and post extension at 72 ºC for 10 min. The PCR products and restriction products were electrophoresis on 2% agarose gel. The amplified PCR product was digested with BslI for GGH -401C>T (126 bp). Digestion pattern of PCR products of GGH -401C>T were CC (126 bp), CT (126,78and48 bp), and TT (78 and 48 bp).

In this study, we used the criteria of common terminology criteria for adverse events version 4.0 (CTCAE) for evaluation of MTX related toxicities (US Department of Health and Human Services, 2009). The toxicities of MTX were composed of anemia, leukopenia, thrombocytopenia, elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT), mucositis, vomiting, and diarrhea. The severity of adverse effects was graded from 0 to 4.

Statistical analysis

The Fishers exact or Chi-square test was used for comparison of genotype and allele frequencies between patients and controls and comparison of MTX related toxicities between wild type and other genotypes. The Odds ratio (OR) with 95% confidence interval (95%CI) was used for estimating the risk of developing MTX related toxicities of each genotypes or alleles. The SPSS 16 software package was used for the statistical calculation and $P<0.05$ was considered statistically significance.

Results

There were 105 children with ALL and 100 controls included in this study. The characteristics of patients are shown in Table 1. The genotype and allele frequencies of DHFR 829C>T and GGH-401C>T in patients and controls are shown in Table 2. There was no statistical difference of genotype or allele frequencies of these polymorphisms between patients and controls.

For the polymorphisms investigated, Tables 3 and 4 show the risk evaluation of developing MTX related toxicities when we compared the presence (grade 2-4 and grade 3-4) of toxicities between children with wild types

Table 1. Characteristics of the Patients

Characteristics		
Median age, years (range)		5.4 (0.41-14.83)
Gender, n (%)	-Male	64 (61.0)
	-Female	41 (39.0)
ALL, n (%)	-B lineage	91 (86.7)
	-T lineage	13 (12.4)
	-Mixed	1 (0.9)
Risk, n (%)	-Low	48 (45.7)
	-Standard	45 (42.9)
	-High	12 (11.4)
MTX dosage, n (%)	-2.5 g/m^2	48 (45.7)
	-5 g/m^2	57 (54.3)

ALL, acute lymphoblastic leukemia; MTX, methotrexate

Table 2. Genotype and Allele Frequencies of DHFR 829C>T and GGH -401C>T in Children with ALL (n=105) and Controls (n=100)

Genotypes	Cases (%)	Controls (%)	p-value	Alleles	Cases (%)	Controls (%)	p-value
DHFR829C>T				DHFR829C>T			
CC	6	14		C	53	57	
CT	94	86	0.099	T	47	43	0.67
TT	0	0	-				
GGH-401C>T				GGH -401C>T			
CC	51	57		C	74	76.5	
CT	46	39	0.42	T	26	23.5	0.87
TT	3	4	1				

The p-value was obtained by chi-square test

Table 3. Association Between Methotrexate Related Toxicity and DHFR 829C>T Polymorphism

Toxicity	Genotype		OR (95%CI)	p-value	Genotype		OR (95%CI)	p-value
	CC(n=6)	CT(n=99)			CC(n=6)	CT(n=99)		
	Toxicity grade 2-4 n(%)	Toxicity grade 2-4 n(%)			Toxicity grade 3-4 n(%)	Toxicity grade 3-4 n(%)		
Anemia	4 (66.7)	86 (86.9)	3.31 (0.55-19.91)	0.203[a]	3 (50)	45 (45.5)	0.83 (0.16-4.33)	1.000[a]
Leukopenia	5 (83.3)	92 (92.9)	2.63 (0.27-25.71)	0.386[a]	5 (83.3)	67 (67.7)	0.42 (0.05-3.73)	0.662[a]
Thrombocytopenia	3 (50)	40 (40.4)	0.68 (0.13-3.53)	0.687[a]	2 (33.3)	27 (27.3)	0.75 (0.13-4.33)	0.667[a]
Acute hepatitis	1 (16.7)	51 (51.5)	5.31 (0.59-47.13)	0.205[a]	1 (16.7)	30 (30.3)	2.17 (0.24-19.41)	0.668[a]
Neutropenia	6 (100)	94 (94.9)	0.68 (0.13-3.53)	0.687[b]	5 (83.3)	86 (86.9)	1.32 (0.14-12.24)	0.586[a]
Vomiting	1 (16.7)	9 (9.1)	0.50 (0.05-4.76)	0.460[a]	0 (0)	1 (1.1)	-	1.000[b]
Diarrhea	0(0)	7 (7.1)	-	1.000[b]	0 (0)	2 (2.1)	-	1.000[b]
Mucositis	3 (50)	17 (17.2)	0.21 (0.04-1.12)	0.082[a]	0 (0)	0 (0)	-	-
Toxic nephropathy	0 (0)	0 (0)	-	-	0 (0)	0 (0)	-	-

[a]p-Value with the chi-square test; [b]p-Value with the Fisher's exact test; OR, Odds ratio; 95% CI, 95% confidence interval

Table 4. Association Between Methotrexate Related Toxicity and GGH-401C>T Polymorphism

Toxicity	Genotype		OR (95%CI)	p-value	Genotype		OR (95%CI)	p-value
	CC (n=54)	CT+TT (n=51)			CC (n=54)	CT+TT(n=51)		
	Toxicity grade 2-4 n(%)	Toxicity grade 2-4 n(%)			Toxicity grade 3-4 n(%)	Toxicity grade 3-4 n(%)		
Anemia	44 (81.5)	46 (90.2)	2.09 (0.66-6.61)	0.319[a]	22 (40.7)	26 (51)	1.51 (0.69-3.27)	0.392[a]
Leukopenia	49 (90.7)	48 (94.1)	1.63 (0.37-7.21)	0.717[a]	23 (52.3)	39 (76.5)	2.97 (1.24-7.13)	0.024[a]
Thrombocytopenia	15 (27.8)	28 (54.9)	3.17 (1.41-7.13)	0.009[a]	8 (14.8)	21 (41.2)	4.02 (1.58-10.26)	0.005[a]
Acute hepatitis	27 (50)	25 (49)	1.13 (0.52-2.42)	0.916[a]	17 (31.5)	14 (27.4)	0.82 (0.36-1.91)	0.812[a]
Neutropenia	52 (96.3)	48 (94.1)	0.62 (0.09-3.84)	0.672[a]	48 (88.9)	43 (84.3)	0.67 (0.22-2.09)	0.688[a]
Vomiting	3 (5.6)	8 (15.7)	3.16 (0.79-12.67)	0.169[a]	2 (3.7)	1 (2)	-	0.486[b]
Diarrhea	3 (5.6)	4 (7.8)	1.45 (0.31-6.81)	0.711[a]	2 (3.7)	0 (0)	-	0.496[b]
Mucositis	7 (13.7)	13 (25.5)	2.15 (0.78-5.94)	0.212[a]	0 (0)	0 (0)	-	-
Toxic nephropathy	0 (0)	0 (0)	-	-	0 (0)	0 (0)	-	-

[a]p-Value with the chi-square test; [b]p-Value with the Fisher's exact test; OR, Odds ratio; 95% CI, 95% confidence interval

Table 5. Allele Frequencies of DHFR 829C>T and GGH -401C>T in Different Ethnic Groups

	Allele Frequencies (%)				
	Japanese	Caucasian	Mexicans	Han Chinese	Thai
DHFR 829C>T					
C allele	85.8[a]	100[b]	NA	NA	57
T allele	14.2	0			43
GGH -401C>T					
C allele	64.0[1]	69.2[1]	71.4[c]	77.8[1]	74
T allele	36.0	30.8	28.6	22.2	26

[1]database in HapMap; [a](Goto et al, 2001); [b](Prasunkumar et al, 2006); [c](Organista-Nava et al)

and other genotypes of each polymorphism.

We found statistically significant association between GGH -401C>T and leukopenia and thrombocytopenia in children after receiving high dose MTX. Patients having GGH -401 CT or TT genotype had higher risk of grade 2-4 and 3-4 thrombocytopenia with ORs of 3.17 (95% CI, 1.41-7.13) and 4.02 (95% CI, 1.58-10.26), respectively. Furthermore, GGH-401 CT and TT genotype was significantly associated with grade 3-4 leukopenia (p=0.024). The association between DHFR 829C>T and MTX toxicities was not demonstrated in this study.

Discussion

To demonstrate the effect of genetic variations involved in MTX metabolism, we selected two candidate genes encoded important enzymes of the MTX metabolic pathway. The candidate polymorphisms of DHFR and GGH genes, used in this cohort, were translated into different amino acids from wild types leading to functional changes (Morandi et al, 1982; Chen et al, 1984; Yao et al, 1996; ;Yin et al, 1999; Chave et al, 2000; Goto et al, 2001; Chave et al, 2003; Dervieux et al, 2004). Therefore, the alterations of their functions may cause variation of MTX efficacy and toxicity.

In this study, we demonstrated that children with ALL who had GGH -401CT or TT genotype were at higher risk of grade 3-4 leukopenia and thrombocytopenia than CC genotype. There were a few studies regarding the effect of this GGH polymorphism on side effects and efficacy of MTX in patients with rheumatoid arthritis (RA) and cancer (Dervieux et al, 2006; Organista-Nava et al., 2010). The GGH -401CC genotype increased risk of MTX related toxicities in RA patients (Dervieux et al, 2006). The GGH -401CT and TT genotype significantly increase risk of relapse compared to CC genotype in a study of children with ALL (Organista-Nava et al., 2010). However, our data was not consistent with these reports. Because of several enzymes involved in MTX metabolism, the genetic variations of other enzymes may influence the results in studies which had different factors such as dosage of MTX and ethnicities. Therefore, the effect of GGH -401C>T on MTX therapy in ALL patients deserves to be further studied.

Our data emphasized that each ethnicities had unique allele frequencies of DHFR829C>T and GGH -401C>T. Thai population had different allele frequencies from other ethnic groups (Table 5). Therefore, the effect of each SNPs on the outcome of diseases may be seen in one population

but not in the others.

In conclusion, our study indicated that the GGH -401CT and TT genotypes were associated with severe leukopenia and thrombocytopenia in children with ALL receiving high dose MTX during consolidation therapy. However, this result needs to be confirmed in the larger cohort and other ethnics so that we may test this polymorphism before MTX administration to prevent severe toxicities.

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