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Frequency distribution of five SNPs in human *GGH* gene and their effects on clinical outcomes of Chinese pediatric patients with acute lymphoblastic leukemia

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Methotrexate (MTX) is widely used in the treatment of childhood acute lymphoblastic leukemia (ALL). Gamma-glutamyl hydrolase (GGH) plays an important role in the disposition of MTX. The aim of this study was to investigate the frequency distribution of five SNPs in the human *GGH* gene and their effects on serum MTX concentrations and clinical outcomes in Chinese children with ALL. Genotyping of 149 pediatric patients for *GGH* rs11545078 C>T, rs11545077 G>A, rs1800909 T>C, rs11545076 T>G, and rs3758149 C>T was performed using the Sequenom MassARRAY system. Serum MTX concentrations were determined using a fluorescence polarization immunoassay. The five SNPs studied were in strong linkage. The minor allele frequencies for rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 were 5.3, 15.0, 14.3, 15.0, and 15.0%, respectively. Four haplotypes (CGTTC, CACGT, TACGT, and TATGT) were observed at frequencies of 84.9, 9.8, 4.5, and 0.8%, respectively. The median C/D ratios of serum MTX at 24 h and 42 h in children with variant haplotypes (12.30 and 0.08 μmol/L per g/m^2, respectively) were higher than those in wild haplotype carriers (11.85 and 0.07 μmol/L per g/m^2, respectively). The event-free survival of patients with variant haplotypes (89.2%) was significantly better than that of patients with wild haplotypes (71.9%, $P< 0.05$). The relapse rate in children with variant haplotypes (8.1%) was lower than that in children with wild haplotypes (15.6%). These findings have implications for the efficacious use of MTX in childhood ALL patients.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer (Hunger and Mullighan 2015; Pui and Evans 2006). The annual incidence of ALL is approximately 3 cases per 100,000 individuals worldwide (Inaba et al. 2013). In Chinese children under 15 years old, the incidence is approximately 5 cases per 100,000 individuals (Yeoh et al. 2013). Due to advances in chemotherapy, the 5-year survival rate has approached 80% in children with ALL (Pui and Evans 2013). However, many children experience relapse or progression of the disease. The different clinical outcomes can in part be explained by genetic variability in the targets, transporters, and metabolizing enzymes of chemotherapy drugs (Cheok et al. 2009; Moradveisi et al. 2019; Schmiegelow 2009).

Methotrexate (MTX) is an important component of chemotherapy for childhood ALL (Winter et al. 2018). It exerts an anti-leukemic effect by inhibiting dihydrofolate reductase and interfering with DNA synthesis (Jolivet et al. 1983). The non-specific anti-metabolic effects of MTX could possibly disturb normal organ function. Myelosuppression, hepatotoxicity, and mucositis are among the common adverse effects of MTX treatment, which often result in dose reduction or early termination of treatment. This is one reason for the progression or relapse of ALL patients (Oosterom et al. 2018; Vaishnavi et al. 2018). There is a strong correlation between MTX exposure and pharmacological effects (Yang et al. 2018). To minimize the side effects of MTX, drug concentrations are often set between 20 - 100 μmol/L at 24 h and below 1.0 μmol/L 48 h after administration. It would be useful to identify predictors of the pharmacokinetics and adverse effects of MTX in pediatric patients with ALL.

Gamma-glutamyl hydrolase (GGH) is an important lysosomal glycoprotein involved in the disposition of MTX (Schneider and Ryan 2006). It is responsible for removal of γ-glutamate residues from MTX polyglutamates (MTXPGs). Polyglutamates are the active forms of MTX in leukemia cells, and higher concentrations of MTXPGs are associated with greater anti-leukemic effects (Masson et al. 1996). GGH converts the long-chain MTXPGs to short-chain MTXPGs, and the short-chain MTXPGs are more easily effluxed from leukemia cells. Therefore, higher GGH activity could reduce the anti-leukemic effects of MTX. Indeed, increased GGH activity was found in acute myeloid leukemia cells resistant to MTX (Rots et al. 1999). Human GGH activity is influenced by single nucleotide polymorphisms (SNPs) in the *GGH* gene. However, there are few pharmacogenetic studies on SNPs of the *GGH* gene. The aim of the present study was to investigate the frequency distribution of five SNPs in the *GGH* gene and determine their effects on serum MTX concentrations and clinical outcomes in Chinese children with ALL.

2. Investigations and results

2.1. Clinical characteristics of children with ALL

One hundred and thirty-three pediatric patients (80 male/53 female) were evaluable and included in the analysis. Sixteen patients were not evaluable because genotype information for the five investigated SNPs (rs11545078 C>T, rs11545077 G>A, rs1800909 T>C, rs11545076 T>G, and rs3758149 C>T) was not complete. The clinical characteristics of the children included in the study are summarized in Table 1. The majority of the study population were treated for B-cell ALL (n = 103). The median age of included patients was 7 years (range 1–17 years).

Table 1: Patient demographics and clinical characteristics

Characteristics		Mean ± SD	Median	Range
Age (y)		7.50 ± 4.06	7	1 - 17
Gender, % (n)	Male	60.2 (80)		
	Female	39.8 (53)		
ALL immunotype, % (n)	B lineage	77.4 (103)		
	T lineage	9.8 (13)		
	Mixed	1.5 (2)		
	Unkown	11.3 (15)		
Risk, % (n)	Standard	61.6 (82)		
	Middle	30.1 (40)		
	High	8.3 (11)		
MTX dose (g/m^2)		2.46 ± 0.39	2.50	2.00 - 4.00
MTX C_{24}		31.39 ± 13.85	29.50	10.17 - 93.67
MTX C_{42}		0.41 ± 1.16	0.18	0.00-9.77

Abbreviations: ALL, acute lymphoblastic leukemia; MTX, methotrexate; C_{24}, concentration at 24 h; C_{42}, concentration at 42 h.

2.2. Genotyping analysis of five SNPs in the GGH gene

The genotype and allele frequencies of the five investigated SNPs in the *GGH* gene are displayed in Table 2. The genotype distribution for the five SNPs was in Hardy-Weinberg equilibrium (P > 0.05). The minor allele frequencies for rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 were 5.3, 15.0, 14.3, 15.0, and 15.0%, respectively. The results of linkage disequilibrium analysis are shown in Fig. 1. The five SNPs studied were in strong linkage. The standardized linkage disequilibrium coefficient (D') was 0.82 between rs11545078 and rs11545077. The D' for the remaining SNP combinations was 1. Four haplotypes (CGTTC,

Table 2: Genotypes and allele frequencies for *GGH* rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 in Chinese children with ALL

SNP	Genotype frenquency % (n)			Allele frenquency % (n)	
rs11545078	CC	CT	TT	C	T
	90.2 (120)	9.0 (12)	0.8 (1)	94.7 (252)	5.3 (14)
rs11545077	GG	GA	AA	G	A
	72.2 (96)	25.6 (34)	2.2 (3)	85.0 (226)	15.0 (40)
rs1800909	TT	TC	CC	T	C
	73.7 (98)	24.1 (32)	2.2 (3)	85.7 (228)	14.3 (38)
rs11545076	TT	TG	GG	T	G
	72.2 (96)	25.6 (34)	2.2 (3)	85.0 (226)	15.0 (40)
rs3758149	CC	CT	TT	C	T
	72.2 (96)	25.6 (34)	2.2 (3)	85.0 (226)	15.0 (40)
Haplotype: rs11545078/rs11545077/rs1800909/rs11545076/rs3758149					
	CGTTC	CACGT	TACGT	TATGT	
	84.9 (226)	9.8 (26)	4.5 (12)	0.8 (2)	
Genotype: rs11545078/rs11545077/rs1800909/rs11545076/rs3758149					
	CGTTC/CGTTC	CGTTC/CACGT	CGTTC/TACGT		
	72.2(96)	18.0(24)	6.0(8)		
	CGTTC/TATGT	CACGT/TACGT	TACGT/TACGT		
	1.5(2)	1.5(2)	0.8(1)		

Abbreviations: GGH, gamma-glutamyl hydrolase; ALL, acute lymphoblastic leukemia.

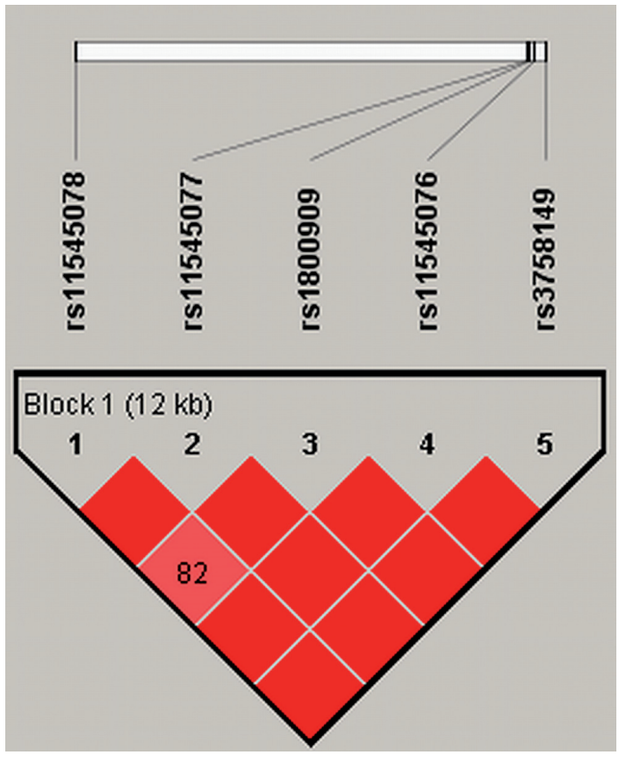

Fig.: Linkage disequilibrium map of selected SNPs (rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149) in the human ***GGH*** gene.
Abbreviations: GGH, gamma-glutamyl hydrolase; SNP, single nucleotide polymorphism.

Table 3: Effects of *GGH* rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 polymorphisms on MTX C/D ratios at 24 h and 42 h after administration in Chinese children with ALL

			C/D, µmol/L per g/m^2	
	Genotype	n	24 h	42 h
rs11545078	CC	120	11.88 [4.51-46.84]	0.07[0.00-4.89]
	CT+TT	13	13.46 [5.35-22.31]	0.08 [0.00-0.18]
	P-value		0.43	0.62
rs11545077	GG	96	11.85 [4.51-46.84]	0.07[0.00-4.89]
	GA+AA	37	12.30 [5.35-28.44]	0.08 [0.00-0.60]
	P-value		0.34	0.72
rs1800909	TT	98	11.72 [4.51-46.84]	0.07[0.00-4.89]
	TC+CC	35	13.02 [5.35-28.44]	0.08 [0.00-0.60]
	P-value		0.19	0.89
rs11545076	TT	96	11.85 [4.51-46.84]	0.07[0.00-4.89]
	TG+GG	37	12.30 [5.35-28.44]	0.08 [0.00-0.60]
	P-value		0.34	0.72
rs3758149	CC	96	11.85 [4.51-46.84]	0.07[0.00-4.89]
	CT+TT	37	12.30 [5.35-28.44]	0.08 [0.00-0.60]
	P-value		0.34	0.72
Haplotype	CGTTC/CGTTC	96	11.85 [4.51-46.84]	0.07[0.00-4.89]
	Other haplotypes	37	12.30 [5.35-28.44]	0.08 [0.00-0.60]
	P-value		0.34	0.72

Data are given as median (range).
P-value was determined by Mann-Whitney test.
Abbreviations: GGH, gamma-glutamyl hydrolase; MTX, methotrexate; ALL, acute lymphoblastic leukemia.

Table 4: Effects of *GGH* rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 polymorphisms on EFS and RR in Chinese children with ALL

SNP	Genotype	n	EFS		RR	
			Odds ratios (95% CI)	*P*-value	Odds ratios (95% CI)	*P*-value
rs11545078	CC	120	1 (Reference)		1 (Reference)	
	CT+TT	13	0.57 (0.12 - 2.73)	0.73	0.50 (0.06 - 4.14)	1.00
rs11545077	GG	96	1 (Reference)		1 (Reference)	
	GA+ AA	37	0.31 (0.10 - 0.96)	0.04	0.48 (0.13 - 1.75)	0.40
rs1800909	TT	98	1 (Reference)		1 (Reference)	
	TC+CC	35	0.34 (0.11 - 1.05)	0.06	0.52 (0.14 - 1.914)	0.40
rs11545076	TT	96	1 (Reference)		1 (Reference)	
	TG+GG	37	0.31 (0.10 - 0.96)	0.04	0.48 (0.13 - 1.75)	0.40
rs3758149	CC	96	1 (Reference)		1 (Reference)	
	CT+TT	37	0.31 (0.10 - 0.96)	0.04	0.48 (0.13 - 1.75)	0.40
Haplotype	CGTTC/CGTTC	96	1 (Reference)		1 (Reference)	
	Other haplotypes	37	0.31 (0.10 - 0.96)	0.04	0.48 (0.13 - 1.75)	0.40

Abbreviations: GGH, gamma-glutamyl hydrolase; EFS, event-free survival; RR, relapse rate; event-free survival; ALL, acute lymphoblastic leukemia.

CACGT, TACGT, and TATGT) were observed in the 133 patients in the study. The haplotype frequencies were 84.9, 9.8, 4.5, and 0.8%, respectively.

2.3. Effects of the five SNPs in the GGH gene on serum MTX concentrations

The dose-adjusted concentrations (C/D ratios) of serum MTX at 24 h and 42 h after administration in the different genotype groups are shown in Table 3. For the five SNPs investigated, the median C/D ratios of serum MTX at 24 h and 42 h in children with variant genotypes were higher than those in homogeneous wild genotype carriers. However, the differences were not of statistical significance.

2.4. Effects of the five SNPs in the GGH gene on the event-free survival (EFS) and relapse rate (RR)

The distribution of risk stratification was similar between wild genotype and variant genotype groups. As shown in Table 4, the EFS in patients with rs11545077 GA/AA, rs11545076 TG/GG, and rs3758149 CT/TT genotypes (89.2%) was significantly higher than that in patients with rs11545077 GG, rs11545076 TT, and rs3758149 CC genotypes (71.9%, $P< 0.05$). The EFS in patients with rs11545078 CT/TT and rs1800909 TC/CC genotypes (84.6% and 88.6%, respectively) was also higher than that in patients with rs11545078 CC and rs1800909 TT genotypes (75.8% and 72.4%, respectively), but the differences were not of statistical significance. The EFS in patients with variant haplotypes (89.2%) was significantly higher than that in patients with CGTTC/CGTTC haplotypes (71.9%, $P< 0.05$). The RR in children with variant genotypes was lower than that in homogeneous wild genotype carriers, and the RR in children with variant haplotypes (8.1%) was lower than that in children with CGTTC/CGTTC haplotypes (15.6%). There was no statistical significance in the differences of RR.

3. Discussion

MTX is a key drug used in consolidation and maintenance therapies for ALL (Jolivet et al. 1983; Winter et al. 2018). High inter-individual variability has been observed in the pharmacokinetics and efficacy of MTX (Giletti et al. 2017). Approximately 20% of children with ALL do not achieve satisfactory responses to MTX treatment (Pui and Evans 2013). Different pharmacological responses to MTX could be partly explained by genetic variations in metabolizing enzymes, transporters, and targets (Giletti et al. 2017). The enzyme GGH plays an important role in the disposition of MTX and several SNPs have been found in the coding region and promoter of this gene. In the current study, we examined the frequency distribution of five functional SNPs (rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149) in the *GGH* gene and their associations with serum MTX concentrations and clinical outcomes in a cohort of Chinese children with ALL.

One major finding from our study was that the five investigated SNPs in the *GGH* gene were firstly confirmed to be in high linkage disequilibrium in Chinese children with ALL. Of the 133 patients with complete genotype information, three SNPs (rs11545077, rs11545076, and rs3758149) were in complete linkage disequilibrium. These results indicate that they were qualified as Tap SNPs in genome-wide association studies. CGTTC was found to be the most common haplotype. This finding is supported by Garcia-Bournissen et al. (2007). As expected, there were inter-ethnic differences in the distributions of *GGH* polymorphisms between Oriental and Western populations. The MAFs of the five SNPs investigated in our study were similar to those reported in Asian populations (Hashiguchi et al. 2016; Hayashi et al. 2009; Yamamoto et al. 2016) but lower than those reported in Caucasian populations (Dervieux et al. 2004; DeVos et al. 2008; Hegyi et al. 2017).

Cheng et al. (2004) reported that the rs11545078 C>T polymorphism produces an amino acid substitution of threonine to isoleucine in exon 5 of the human *GGH* gene and is associated with high GGH activity in leukemia cells from patients treated with MTX. Hattinger et al. (2016) found that the rs11545078 C>T polymorphism is associated with EFS in patients with high-grade osteosarcoma. The rs1800909 T>C polymorphism results in a cysteine to arginine residue substitution. They also found that the rs1800909 T>C polymorphism is associated with an increased risk for MTX toxicity. However, van der Straaten et al. (2007) did not see clinical importance of rs11545078 C>T and rs1800909 T>C polymorphisms for MTX treatment outcomes in patients with rheumatoid arthritis. As seen in the dbSNP database, the rs11545077 A>G polymorphism is also a missense variant, which leads to an alanine to serine residue change in the protein sequence. However, no published functional studies were found for this SNP. The rs11545076 G>T and rs3758149 C>T polymorphisms are both located in the promoter of the *GGH* gene, and Chave et al. (2003) showed that both SNPs enhance promoter activities in HepG2 and MCF-7 cells. However, in our previous studies, we observed that, compared with the rs3758149 T allele, the C allele has higher transcriptional activity and stronger binding affinity for nuclear protein extracts in CEM/C1 cells (Wang et al. 2019). Koomdee et al. (2012) found that patients with rs3758149 CT and TT genotypes have an increased risk of leukopenia and thrombocytopenia after MTX chemotherapy. In our study, no significant associations

were found between the investigated SNPs in the *GGH* gene and C/D ratios of MTX. This indicates that these SNPs have no major impact on serum MTX levels. However, we observed that patients carrying variant alleles have higher serum MTX levels and better EFS and RRs than wild type carriers, suggesting the studied SNPs might have potential as efficacy biomarkers of MTX chemotherapy in ALL patients. Since these five SNPs are in the same linkage disequilibrium block, the observed variations in MTX levels, EFS, and RRs could be due to the haplotype or the SNPs alone.

There were some limitations of our study. First, the small sample size in this single center study may have led to bias in the interpretation of the study results. Secondly, the fluorescence polarization immunoassay used in this study did not have the specificity required to distinguish MTX from its derivatives, such as MTXPGs. Third, data on toxicities of MTX treatment were not collected in the current study. Additional research is needed to evaluate the effects of these SNPs on toxicities of MTX chemotherapy in our population.

In summary, the present study confirmed that *GGH* rs11545078, rs11545077, rs1800909, rs11545076, and rs3758149 are in high linkage disequilibrium. Compared with wild type carriers, patients with variant alleles had higher serum MTX levels and better EFS and RRs. These findings have implications for the efficacious use of MTX in childhood ALL patients.

4. Experimental

4.1. Subjects

The study population was composed of 149 ALL patients treated at the Pediatric Department of Beijing Shijitan Hospital, Capital Medical University, Beijing, China. All patients received a high dose of MTX with leucovorin rescue as their maintenance therapy. Clinical information was extracted from the electronic medical record system. The study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University and was performed in accordance with the 1964 Declaration of Helsinki and its later amendments. All children or their parents gave informed consents before participation in the study.

4.2. MTX concentration determination

Venous blood samples were collected from all children at 24 h and 42 h after the start of MTX infusion. Serum MTX concentrations were measured using a fluorescence polarization immunoassay on a TDxFLx analyzer (Abbott Laboratories, Abbott Park, IL, USA). The lower limit of detection of this assay was 0.01 μmol/L. The accuracies of quality control samples ranged from 90 - 110%. The intra- and inter-day assay variations were all below 10%. The C/D ratios of MTX were calculated by dividing the concentrations (μmol/L) by the corresponding 24-h doses (g/m^2).

4.3. Genotyping

Genomic DNA was extracted from 200 μL of blood sample using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA concentration and purity were measured using absorbance values at 260 nm and 280 nm. Five SNPs (rs11545078 C>T, rs11545077 G>A, rs1800909 T>C, rs11545076 T>G, and rs3758149 C>T) were genotyped using the Sequenom MassARRAY system (Sequenom iPLEX Assay, San Diego, CA, USA). Primers for polymerase chain reaction amplification and extension were designed using MassARRAY Assay Design 3.1 software (Sequenom). Detection of specific alleles was achieved by matrix-assisted laser desorption/ionization time of flight mass spectrometry. The genotyping call rates for the five investigated SNPs (rs11545078 C>T, rs11545077 G>A, rs1800909 T>C, rs11545076 T>G, and rs3758149 C>T) were 97.3, 100, 92.6, 92.6, and 100%, respectively.

4.4. Statistical analysis

Graphpad Prism Software version 4.0 was used for all statistical analyses. Continuous data are expressed as mean ± SD or median (range). Categorical data are expressed as number and percentage. The genotype and allele frequencies were assessed for deviation from Hardy–Weinberg equilibrium using the chi-squared test. Linkage disequilibrium between each pair of SNPs was determined using HaploView software. The Mann-Whitney test was used for comparisons of the C/D ratios of MTX between two non-normally distributed groups. Differences of EFS and RR based on genotype were compared using a log-rank test. $P < 0.05$ was considered statistically significant.

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Conflicts of interest: All authors declare not to have competing interests.

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