Influence of dihydrofolate reductase gene polymorphisms rs408626 (-317A>G) and rs442767 (-680C>A) on the outcome of methotrexate-based maintenance therapy in South Indian patients with acute lymphoblastic leukemia

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Abstract

Purpose The most common cause of treatment failure in acute lymphoblastic leukaemia (ALL) is the relapse. Genetic polymorphisms of dihydrofolate reductase (DHFR) enzyme affect the response to methotrexate (MTX) treatment. Inter-individual variability exists in the distribution of DHFR variants, and they influence MTX treatment outcome. To the best of our knowledge, there are no genetic studies reported from India, which have explored the influence of DHFR variants on the outcome of MTX treatment. Therefore, we aim to study the influence of DHFR rs408626 (-317A>G) and rs442767 (-680C>A) variants on ALL outcome in South Indian patients.

Methods A total of 70 ALL patients who were on MTX-based maintenance therapy were recruited for the study. DNA was extracted from leukocytes, and genotyping was done by real-time PCR.

Results The DHFR-317GG genotype was associated with the increased risk of relapse in patients with ALL (relative risk 2.25, 95 % confidence interval (CI) 1.38 to 3.6, $p = 0.02$).

DHFR-317AA and -680CA genotypes were found to be associated with severe leucopenia ($p < 0.05$). In Cox regression model, -317GG genotype was found to have lower relapse-free survival (hazard ratio (HR) 2.56, 95 % CI 1.06 to 6.19, $p = 0.03$) and overall survival (HR 3.72, 95 % CI 1.44 to 9.65, $p = 0.007$). Similarly, patients with white blood cell (WBC) count >50,000 cells/mm^3 were also found to have lower relapse-free survival (HR 2.20, 95 % CI 1.10 to 4.79, $p = 0.04$) and overall survival (HR 3.30, 95 % CI 1.45 to 7.53, $p = 0.004$).

Conclusion The GG genotype of *DHFR*-317A>G variant is associated with increased risk of ALL relapse and lower overall survival in South Indian population. Both variants of *DHFR* (-317 AA and -680 CA) are found to be associated with severe leucopenia caused by MTX.

Keywords Acute lymphoblastic leukaemia · *DHFR* · South Indians · Methotrexate

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Introduction

Acute lymphoblastic leukaemia (ALL) is a haematological malignancy characterized by an uncontrolled proliferation of lymphoblasts. Accumulation of blasts hinders the production of cellular components of blood. Although it affects all age groups, it is the most frequent form of childhood cancers [1, 2]. A total number of 5730 new ALL cases were reported from the USA in 2011, and around 1420 deaths occur per year in the USA that are due to ALL [3]. Population-based data from the Indian cancer registries suggest that approximately 10,000 new cases are being diagnosed each year [4]. Since the introduction of systemic chemotherapy, cure rates of childhood ALL have improved from less than 10 % in the 1960s to 80 % today in western countries [5, 6]. Despite high cure rates,

the annual number of children relapsing after initial therapy remains greater than new cases of various childhood cancers [7]. Moreover, relapsed ALL is the fourth most common childhood malignancy [8]. The relapse rate in North and South India were found to be 30 and 40 %, respectively [4, 9]. Observational and clinical studies indicate 6-mercatopurine/ methotrexate (MTX) maintenance therapy to be superior to other drug combinations [10, 11]. A systematic review of 42 randomized studies with 12,000 childhood ALL cases indicated that longer maintenance therapy was associated with a slightly lower risk of relapse [11]. Data from our regional cancer registries suggest that despite following 6MP/MTX combination during maintenance therapy at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), the percentage of relapse cases are high (40 %) during maintenance therapy even after achieving complete remission during the induction and consolidation phases of ALL treatment. MTX, an essential component of ALL treatment protocols, exerts its anticancer activity by inhibiting the enzyme dihydrofolate reductase (DHFR). In both experimental and clinical settings, altered levels of DHFR or decreased DHFR–MTX complex formation are found to be associated with relapse and MTX resistance [12–15]. Many drugs metabolizing enzymes are known to be genetically polymorphic, which leads to inter-individual variations in drug response [16, 17]. Hence, polymorphisms in genes coding for folate-metabolizing enzymes may serve as predictors of MTX response or toxicity in ALL. Among the folate-metabolizing enzymes, the polymorphisms of genes involved in DNA synthesis such as DHFR (target of MTX) and thymidylate synthase can potentially affect MTX action.

Functional/regulatory polymorphisms in the DHFR gene can affect its function or expression and subsequently can alter sensitivity to MTX [18, 19]. To the best of our knowledge, only three studies exist in the literature where the influences of DHFR promoter polymorphisms on outcome of ALL have been studied. Interestingly, the results of all these studies were contradictory to each other [18, 20, 21]. Discordant results of DHFR promoter variants could be due to a difference in the distribution of DHFR variant alleles, different treatment protocols with different doses of MTX, end points used and sample size. In the previous studies, patients across different phases of ALL treatment protocol (induction, consolidation and maintenance) were considered. There might be an effect of other drugs on the outcome which was given along with MTX during these phases. The high-dose MTX was given across different phases of ALL protocol that might confound the influence of genotype on the outcome of MTX therapy. Since the genotype effects may be more evident when drugs are given at lower doses, only patients receiving low-dose MTX orally during maintenance therapy were considered in the present study. Moreover, the end point of the previous studies was an event, which was defined as either relapse or

death or induction failure. In the present study, the end point is the only relapse of patients who achieved complete remission. Besides, South Indian population represents a genetically distinct group [22]. As the difference in the distribution of alleles could confound genotype-phenotype association studies and are the primary reasons for the conflicting results, it is important to explore the influence of DHFR variants in each population. Therefore, we aimed to evaluate the association of DHFR gene variants rs408626 (-317A>G) and rs442767 (-680C>A) with ALL outcome during MTX-based maintenance therapy in South Indian patients.

Materials and methods

Study population

A prospective cohort study with a sample size of 70 patients of either gender who were on MTX-based maintenance therapy was conducted in the Department of Medical Oncology, JIPMER, from September 2010 to September 2013. We calculated the sample size to be 70 patients with ALL considering a power of 90 %, 5 % type-I error rate, a relative risk (RR) of 2.5, relapse rate of 30 % and minor allelic frequency of 10 % using power and sample size software (version 3.0, 2009). All patients were followed up from the start of maintenance therapy until the occurrence of relapse or death or till the last date of analysis (June 18th, 2015). Patients who were less than 1 year of age, pregnant women, patients with hepatic or renal dysfunction and patients with other malignancies were excluded from the study. The study was approved by the Institute Ethics Committee of JIPMER, Puducherry, India. Written informed consent was obtained from all study participants, and in the case of children, consent was obtained from their legally accepted representatives.

Treatment protocol and end points in the analysis

Patients below 25 years of age were treated using Multicentric protocol-841(MCP) I$_2$A and above 25 years of age were treated with modified GMALL-84 protocols. MTX dose did not differ across both the protocols during maintenance therapy. All patients received 15–20 mg/m^2/week dose of MTX during maintenance therapy for 2 to 3 years. Relapse was defined as the presence of >5 % of malignant blast cells in the bone marrow or on histological or cytological documentation of blasts in extramedullary sites. Relapse-free survival (RFS) was the duration between the start of maintenance therapy and the date of occurrence of relapse or last date of analysis. Overall survival (OS) was the time between the date of diagnosis and date of death or till the last date of analysis. Toxicities were evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE-version 4)

recommended by the National Cancer Institute [23] and included haematopoietic toxicity, as well as non-haematopoietic toxicity (hepatic and renal toxicity). Maintenance phase consisted of 6 cycles and each cycle lasted for 3 months. The highest grade of toxicity during each cycle was recorded. All toxicities recorded during the first three cycles of maintenance phase were considered as early phase toxicity.

DNA extraction and genotyping

Five millilitres of the venous blood was collected in tubes containing 100 μL of 10 % ethylenediamine tetra-acetic acid (EDTA) and centrifuged for 5 min at 2500g. Plasma was discarded and the pellets containing red blood corpuscles with the buffy coat were stored at −20 °C until DNA extraction. Genomic DNA was extracted from the peripheral leucocytes by the phenol–chloroform method. The extracted DNA was analysed qualitatively and quantitatively using biophotometer plus (Eppendorf AG 22331, Hamburg, Germany). Each DNA sample was diluted to an optimal concentration of 50 ng/μL suitable for further downstream analysis and stored in aliquots at 4 °C. Genotyping of DHFR variants was carried out by real-time PCR (7300 Applied Biosystems; Life Technologies Corporation, Carlsbad, CA, USA) using TaqMan SNP genotyping assays [rsid 408626 (-317A>G) and rsid 442767 (-680 C>A) Online source-1] according to manufacturer instructions. The version 1.4 of 7300 sequence detection software (SDS) was used for allelic discrimination. All samples were analysed in duplicates and the results were found to be 100 % concordant.

Statistical analysis

The observed genotype frequencies were tested for Hardy–Weinberg Equilibrium (HWE) using the chi-square test. The proportions of relapse between different genotypes as well as various prognostic factors were compared using the Fisher's exact test and the relative risk (RR) with 95 % confidence interval (CI) is presented. Comparison of early phase and late phase toxicities were done by Fisher's exact test. The association between genotype and toxicity in each cycle of maintenance phase was tested using Fisher's exact test. Univariate binary logistic regression was done to estimate the odds ratio (OR) and CI. Factors or variables whose p value was less than 0.25 in univariate analysis were considered for multivariate binary logistic regression analysis to estimate the percentage of variability in the clinical outcome because of genetic/non-genetic factors. Kaplan–Meier curves were plotted for RFS and OS among different genotypes and other prognostic factors of ALL. Log-rank test was used to test for statistical significance of the above parameters. Univariate Cox regression analysis was used to estimate the hazard ratio (HR) with 95 % CI; multivariate Cox regression was used to assess the

impact of genotypes on clinical outcomes in the presence of other covariates. Statistical analysis of data was performed using GraphPad Instat 3 (GraphPad Software Inc., San Diego, CA, USA) and SPSS software (version 16). The level of statistical significance was set at $p < 0.05$.

Results

Characteristics of the ALL patients

Out of the 70 patients, 28 relapsed. There were 25 females and 45 males in the study. The age (mean ± SD) of relapsers was 14.75 ± 11.13 and that of non-relapsers was 15 ± 12.14. The demographic and clinical characteristics were not significantly different between relapse and non-relapse groups (Table 1). Patients with white blood cell (WBC) count >50,000 cells/mm^3 at the time of diagnosis were at a twofold increased risk of death (RR 1.89, 95 % CI = 1.09 to 3.26, $p = 0.03$; Table 1).

The genotype distribution and allele frequencies of DHFR –317A>G and -680C>A variants in patients with ALL

The percentages of A and G alleles of the DHFR-317 variant were 67.1 and 32.9, respectively, in the study population. The percentages of DHFR-317A>G variant genotypes were as follows: AA = 47.1 %, AG = 40.1 %, GG = 12.8 %, and these were concordant with HWE. The percentage of C and A alleles of -680 variant were 39 and 61, respectively. The CC genotype was found to be absent in our population, and the percentages of AC and AA are 78.6 and 21.4, respectively. DHFR-680C>A variant genotype frequencies were discordant with HWE.

Association between genotype and outcome were tested in different genetic models [24] using Fisher's exact test. The allele and genotype frequencies of *DHFR*-317A>G variant were significantly different between patients with and without relapse and similarly between dead and alive groups in recessive genetic model (RR = 2.25; 95 % CI = 1.38 to 3.6; $p = 0.02$; Table 2). DHFR-680C>A variant was not associated with either relapse or death in ALL patients (Table 2).

In a multivariate regression model that included covariates such as WBC, haemoglobin and hepatosplenomegaly, -317GG genotype remained associated with relapse (Table 3). The model with genotype and non-genetic covariates explained 11 to 21 % of the variability in relapse with DHFR-317GG genotype contributing to 11.4 % of this variability. Similarly -317A>G variant and WBC count remained significantly associated with death in the presence of other known prognostic factors of ALL (Table 3) which explained 11 to 26 % of the variability. DHFR-317GG genotype and WBC contributed 11 and 9 % to this variability, respectively.

Table 1 Comparison of salient clinical and laboratory parameters at the time of diagnosis in patients with ALL

Parameters	Relapse cases, N = 28, n (%)	Non-relapse cases, N = 42, n (%)	RR (95 % CI)	p value	Dead, N = 28, n (%)	Alive, N = 42, n (%)	RR (95 % CI)	p value
Age (years)								
≤9	9 (32.1)	16 (38.1)	0.85 (0.45 to 1.59)	0.79	10 (35.7)	15 (35.7)	0.98 (0.54 to 1.82)	>0.99
>9	19 (67.9)	26 (61.9)			18 (64.3)	27 (64.3)		
Gender								
Males	18 (64.3)	27 (64.3)	0.98 (0.54 to 1.82)	>0.99	21 (75)	24 (57.1)	1.66 (0.82 to 3.3)	0.20
Females	10 (35.7)	15 (35.7)			7 (25)	18 (42.9)		
Lymphadenopathy								
Yes	18 (64.3)	25 (59.5)	1.13 (0.61 to 2.0)	0.80	20 (71.4)	23 (54.8)	1.57 (0.80 to 3.04)	0.21
No	10 (35.7)	17 (40.5)			8 (28.6)	19 (45.2)		
Hepatosplenomegaly								
Yes	23 (82.1)	29 (69)	1.59 (0.71 to 3.56)	0.27	23 (82.1)	29 (69)	1.59 (0.71 to 3.56)	0.27
No	5 (17.9)	13 (31)			5 (17.9)	13 (31)		
Mediastinal mass								
Yes	7 (25)	14 (33.3)	0.77 (0.39 to 1.54)	0.59	9 (32.1)	12 (28.6)	1.10 (0.60 to 2.02)	0.79
No	21 (75)	28 (66.7)			19 (67.9)	30 (71.4)		
WBC (cells/mm^3)								
>50,000	12 (42.9)	10 (23.8)	1.63 (0.94 to 2.84)	0.11	13 (46.4)	9 (21.4)	1.89 (1.09 to 3.26)	0.03*
≤50,000	16 (57.1)	32 (76.2)			15 (53.6)	33 (78.6)		
Hb (g %)								
<11	24 (71.4)	30 (85.7)	1.77 (0.72 to 4.37)	0.24	23 (82.1)	31 (73.8)	1.36 (0.61 to 3.0)	0.56
≥11	3 (14.3)	12 (28.6)			5 (17.9)	11 (26.2)		
Platelets (cells/mm^3)								
<100,000	21 (75)	27 (64.3)	1.37 (0.68 to 2.74)	0.43	21 (75)	27 (64.3)	1.37 (0.68 to 2.74)	0.43
≥100,000	7 (25)	15 (35.7)			7 (25)	15 (35.7)		
D8 blasts								
Yes	3 (10.7)	4 (9.5)	1.08 (0.43 to 2.67)	>0.99	2 (7.1)	5 (11.9)	0.69 (0.20 to 2.37)	0.69
No	25 (89.3)	38 (90.5)			26 (92.9)	37 (88.1)		
Lineage								
B-ALL	14 (50)	21 (50)	–	>0.99	13 (46.4)	22 (52.4)	–	0.86
T-ALL	10 (35.7)	15 (35.7)			11 (39.3)	14 (33.3)		
Not available	4 (14.3)	6 (14.3)			4 (14.3)	6 (14.3)		

The p values were obtained by Fisher's exact test

RR relative risk, 95 % CI 95 % confidence interval

*p value <0.05 is significant

Association of toxicity between early and late phases of maintenance therapy

A total of 64, 57, 51, 45, 42 and 40 patients were considered for the assessment of haematological toxicity in 1, 2, 3, 4, 5 and 6 cycles of maintenance therapy, respectively. We found that grade 3 and 4 haematopoietic toxicities were more in the early phase of maintenance therapy ($p < 0.001$, Table 4).

There was no difference in the liver toxicity between early and late phases of maintenance therapy. Renal toxicity was not observed in the studied population.

Association between DHFR variants and MTX toxicity

We compared the different grades of toxicity between genotypes in each cycle of maintenance therapy. We found that patients with DHFR-317AA genotype are at increased risk of developing grade 3 or 4 toxicity in the second cycle (a part of early phase toxicity) of maintenance phase (RR 3.09, 95 % CI 1.30 to 7.29, $p = 0.005$). Similarly DHFR-680AC genotype was also associated with grade 3 or 4 toxicity in cycle 2 of maintenance treatment (Table 5).

Table 2 The genotype distribution of DHFR (-317A>G and -680C>A) variants and its influence on ALL outcome

Allele and genotypes	Relapsers, $N = 28$, n (%)	Non-relapsers, $N = 42$, n (%)	RR (95 % CI)	p value	Dead, $N = 28$, n (%)	Alive, $N = 42$, n (%)	RR (95 % CI)	p value
DHFR-317A>G								
AA	11 (39.3)	22 (52.4)	–	0.04	14 (50)	19 (45.2)	–	0.01
AG	10 (35.7)	18 (42.9)			7 (25)	21 (50)		
GG	7 (25)	2 (4.8)			7 (25)	2 (4.8)		
Genetic models[a]								
Recessive								
GG	7 (25)	2 (4.8)	Ref	0.02	7 (25)	2 (4.8)	Ref	0.02
AG + AA	21 (75)	40 (95.2)	2.25 (1.38 to 3.6)		21 (75)	40 (95.2)	2.25 (1.38 to 3.6)	
Dominant								
AG + GG	17 (60.7)	20 (47.6)	Ref	0.33	14 (50)	23 (54.8)	Ref	0.80
AA	11 (39.3)	22 (52.4)	1.37 (0.75 to 2.50)		14 (50)	19 (45.2)	0.89 (0.50 to 1.58)	
Co-dominant								
AG	10 (35.7)	18 (43)	Ref	0.80	7	21	Ref	0.05
AA + GG	18 (64.3)	24 (57)	0.86 (0.46 to 1.59)		21	21	0.50 (0.24 to 1.01)	
DHFR-680C>A								
AC	21 (75)	34 (81)	Ref	0.56	21 (75)	34 (81)	Ref	0.56
AA	7 (25)	8 (19)	0.81 (0.43 to 1.54)		7 (25)	8 (19)	0.81 (0.43 to 1.54)	

The p values were obtained by Fisher's exact test. p value <0.05 is significant

Ref reference, *RR* relative risk, *95 % CI* 95 % confidence interval

[a] Genetic models [24]

Association of DHFR-317A>G polymorphism with RFS and OS in patients with ALL

Patients with homozygous mutant genotype (GG) had a median RFS time and OS time of 14 and 24 months, respectively. The RFS (HR = 2.56, 95 % CI = 1.08 to 6.08, p = 0.02) and OS (HR = 2.51, 95 % CI = 1.06 to 5.95, p = 0.02) were found to be lower in patients with GG genotype (Fig. 1a, b).

There was no significant difference in RFS and OS time between -680AA carriers and CC carriers (Fig. 1c, d, p = 0.65). Patients who had WBC count of >50,000 cells/mm^3 at the time of diagnosis had a lower RFS (Fig. 1e, HR = 2.35, CI = 1.09 to 5.0, p = 0.02) and OS (Fig. 1f, HR = 2.5, CI = 1.22 to 5.46, p = 0.02). Genotype and WBC

retained their significance in co-regression, when other prognostic factors were taken into account (Table 6).

Discussion

To the best of our knowledge, this is the first study from India to provide an insight into the influence of *DHFR* promoter polymorphisms (-317A>G and -680C>A) on outcome of MTX-based maintenance therapy in a South Indian population. DHFR plays a crucial role in folate metabolism, and it is an important target of MTX. Mutations in the *DHFR* gene were found to influence susceptibility to various diseases and drug response [19, 25–29]. There is a paucity of the

Table 3 Risk factors for ALL relapse and survival: multivariate binary logistic regression analysis

	Relapse		Survival	
Variables	OR (95 % CI)	p value	OR (95 % CI)	p value
DHFR-317GG	7.80 (1.38 to 43.9)	0.02*	8.32 (1.42 to 48.73)	0.01*
Hepato-splenomegaly	0.56 (0.15 to 2.0)	0.37	1.69 (0.46 to 6.14)	0.42
White blood cell count	2.65 (0.87 to 8.04)	0.08	4.24 (1.33 to 13.50)	0.01*
Haemoglobin	1.70 (0.43 to 6.6)	0.44	–	
Gender	–		2.35 (0.72 to 7.69)	0.15

The p values were obtained by logistic regression

OR odds ratio, *95 % CI* 95 % confidence interval, *DHFR* dihydrofolate reductase, *en dash* indicates not included in the analysis

*p value <0.05 is significant

Table 4 Comparison of haematological toxicity between early and late phases of MTX-based maintenance therapy

	Anaemia				Leucopenia				Thrombocytopenia			
	Severe	Mild	RR (95 % CI)	p value	Severe	Mild	RR (95 % CI)	p value	Severe	Mild	RR (95 % CI)	p value
Early phase	46	74	2.72 (1.66 to 4.48)	<0.01*	69	106	1.44 (1.03 to 2.02)	0.03*	48	124	2. 7(1.54 to 4.81)	<0.01*
Late phase	18	109			34	88			13	114		

The p values were obtained by Fisher's exact test

RR relative risk, 95 % CI 95 % confidence interval

*p value <0.05 is significant

literature on the influence of DHFR promoter polymorphisms on ALL outcome. An unpublished data from our lab showed that frequencies of DHFR-317A>G and -680C>A polymorphisms were comparable to those of South Indian ALL patients. The absence of CC genotype of -680C>A variant resulted in discordant Hardy–Weinberg equilibrium (DHWE). Population stratification as a cause of discordance is unlikely in our subjects given the high prevalence of endogamy [30].

In the present study, we found that DHFR-317GG genotype confers two fold increased risk of ALL relapse ($p = 0.02$, RR = 2.25, 95 % CI = 1.38 to 3.6; Table 2.). Our study results are in agreement with the Gomez-Gomez et al. study on Mexican ALL patients ($N = 70$; OR = 8.55, 95 % CI 1.84–39.70; $p = 0.006$) [20] but contradictory to Dulucq et al. study where -317 polymorphism was not associated with either relapse or death [18]. DHFR-680C>A had no influence either on relapse or death of patients with ALL, similar to the findings of Dulucq et al. [18]. We hypothesize that patients with GG genotype had altered levels of DHFR expression that might have affected the response to MTX treatment. Transcription factor (TF) binding sites for growth factor independence 1 zinc finger protein (Gfi-1b) was predicted in the region spanning rs 408626 (DHFR-317A>G) using MatInspector, Genomatix, Munich, Germany, http://www.genomatix.d [31]. Gfi-1b is abundant in blood cells and bone marrow tissues [32] and is an important regulator of blood cell production [33–36]. Therefore, Gfi-1b may play a major role in haematological

malignancies. However, the impact of the DHFR-317A>G SNPs on the binding pattern of Gfi-1b has not been studied. Further functional studies must be conducted to investigate the binding pattern and its relation to the expression of DHFR.

Age, gender and WBC count were the known prognostic factors of ALL in the western population, but it may vary across developing countries due to various reasons [4].

Patients with ≤9 years of age are known to have a better prognosis than patients who are above 9 years [37]. But in the present study, we did not find any difference between these two groups similar to other studies conducted in India [4, 38]. Initially, we thought this might be due to the inclusion of patients who are older than >24 years of age (13 patients were treated with G-MALL protocol). Hence, we reanalysed the data excluding them from the analysis even then we did not find any association between the groups (Online resource 2, $p = 0.59$). In the present study, the number of males were almost twice that of females similar to the other study from India [38] but in contrast to the figures reported from developed countries [39, 40]. This might be due to the neglect of female children in India and probably the special treatment of male siblings. It has been reported in various studies [24, 26] that females have better survival than males of the same age with ALL. However, we did not observe such a gender-based outcome ($p > 0.05$) (Table 3), similar to Dulucq et al., Kim et al. and Dervieux et al. [18, 41]. This could be due to the small sample size of the female group ($n = 25/70$) in our study.

Table 5 Association of DHFR variants with leucopoenia in the second cycle of maintenance therapy

Genotypes of DHFR	Toxicity grades (leucopenia)		p value
	Severe (grades 3 and 4)	Mild (grades 0, 1 and 2)	
-317A>G			
AA	16	13	0.005*
AG + GG	5	23	
-680C>A			
AC	21	28	0.02*
AA	0	8	

The p values were obtained by Fisher's exact test

*p value <0.05 is significant

Fig. 1 Kaplan–Meier curves showing RFS and OS for patients with ALL according to DHFR variants (-317A>G and -680C>A) and WBC count. **a** Association of DHFR-317A>G variant with RFS. **b** Association of DHFR-317A>G variant with OS. **c** Association of DHFR-680C>A variant with RFS. **d** Association of DHFR-680C>A variant with OS. **e** Association of WBC count with RFS. **f** Association of WBC count with OS. The number of patients in each curve representing the patients with indicated variable, number of individuals with an event (*in the parenthesis*), as well as the *p* value, estimated by log-rank test for the survival differences between the patients' groups, are indicated in the plot
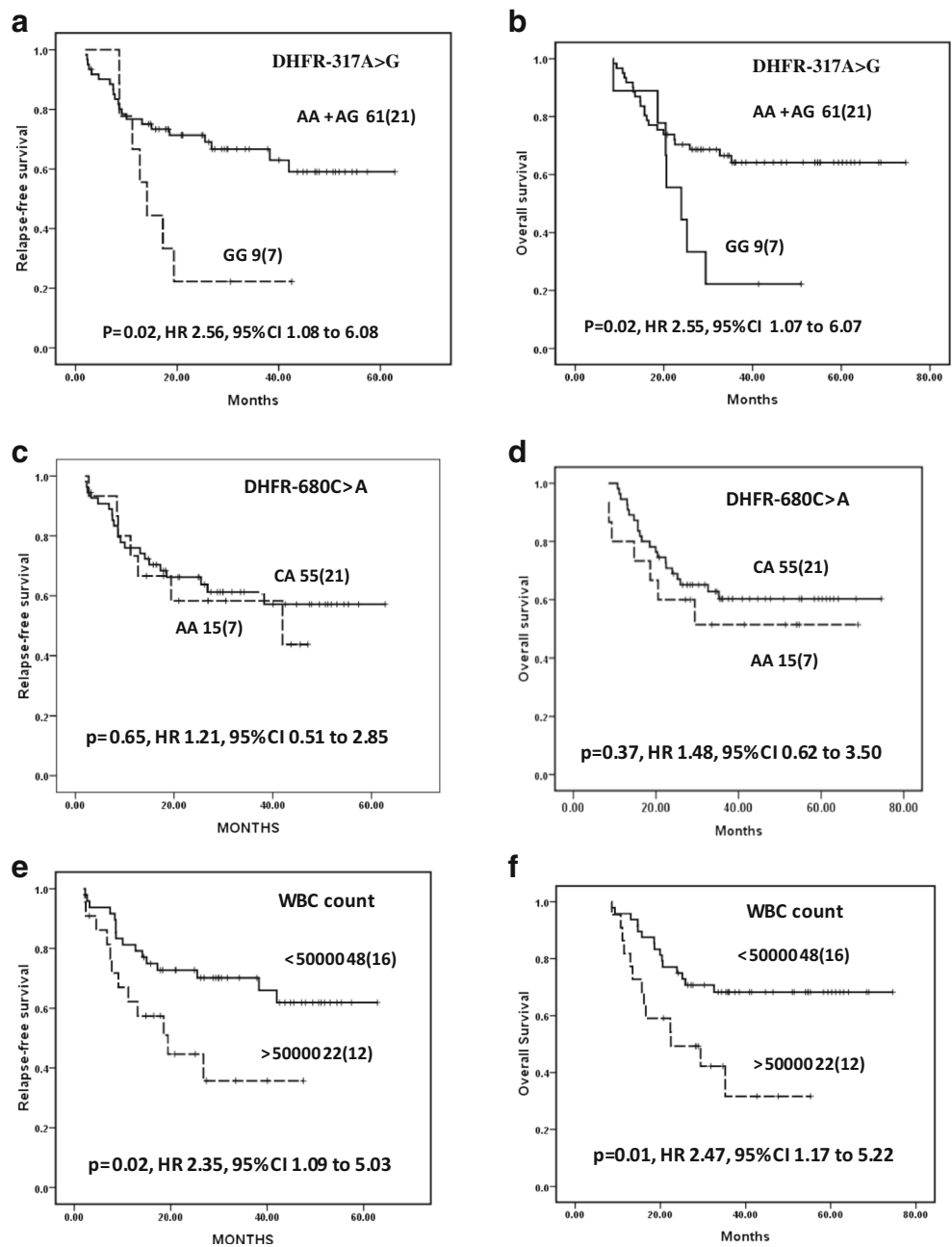
In univariate analysis, the presence of WBC count >50,000 cells/mm^3 showed a trend towards significance in conferring a possible risk of relapse ($p = 0.09$, OR 2.4, 95 % CI 0.85 to 6.73). Failure to attain significance may be due to the exclusion of patients with advanced stage of the disease (induction and consolidation failures) and also the small sample size of our study. In univariate logistic regression analysis, we found that -317GG genotype is associated with both relapse and death (OR = 6.7, CI = 1.27 to 34.9, $p = 0.02$). WBC count was found to be associated with death (OR = 3.17, 95 % CI = 1.1 to 9, $p = 0.03$) which is in accordance with Dulucq et al. study [18]. DHFR-680C>A

did not have any effect on the ALL outcome similar to the other studies [18, 21]. Multiple logistic regression analysis showed that -317GG genotype contributes to 11 % of the variability in relapse compared with non-genetic factors alone (9 %). WBC count and -317GG genotype were found to be associated with death contributing to 11 and 9 % of the variability, respectively. WBC and genotype together contributed to 21 % of the variability in death. Owing to the technical constraints and similar diet habits of the population residing in the area of sample collection, we could not include folate levels as a covariate in the multivariate analysis.

Table 6 Estimation of hazard ratio for RFS and OS of ALL patients: Cox regression analysis

Variable	RFS		OS	
	HR (95 % CI)	p value	HR (95 % CI)	p value
DHFR-317GG	2.56 (1.06 to 6.19)	0.03*	3.72 (1.44 to 9.65)	0.007*
HSM	1.69 (0.62 to 4.59)	0.29	1.32 (0.49 to 3.56)	0.581
HB	0.82 (0.27 to 2.52)	0.73	–	–
WBC	2.20 (1.10 to 4.79)	0.04*	3.30 (1.45 to 7.53)	0.004*
Gender	–	–	2.04 (0.81 to 5.13)	0.127
Lymphadenopathy	–	–	1.64 (0.66 to 4.09)	0.285

The p values and hazard ratio (HR) were obtained by Cox regression analysis

95 % CI, 95 % confidence interval, *en dash* not included in the analysis

*p value <0.05 is significant

We observed that grade 3 or 4 haematological toxicity was more in the early phase of maintenance therapy than in the later phase. This could be due to the adjustment of MTX dose in the early phase, leading to reduced toxicity in the later phase of maintenance therapy. When we tested the association of genotypes with toxicity in each cycle of maintenance, -317AA and -680CA carriers were found to have severe leucopenia (grade 3 or 4) compared to other genotype carriers in the second oral cycle of maintenance therapy.

A study conducted by Salazar et al. on ALL patients who were on consolidation treatment reported that -317A>G and 19-bp ins/del polymorphisms in DHFR gene were in linkage disequilibrium ($r^2 = 0.956$). Allele A of -317 variant contained an insertion while G residue contained a deletion in the 19-bp ins/del polymorphism. It was reported that patients with 19-bp ins/ins, who were also the carrier of -317AA genotype, experienced severe thrombocytopenia (HR 4.7, 95 % CI 1.8 to 12.6, $p = 0.002$). The same study reported that CC genotype of -680C>A was associated with neutropenia (HR 2.9, 95 % CI 1.3 to 7.1, $p = 0.01$) [21]. In contrast to our results, Dulucq et al. did not find any association between -317A>G and -680C>A variants of DHFR and toxicity in ALL [18]. This discrepancy could be due to the use of different doses of MTX across induction, consolidation (high-dose MTX) and maintenance phases (low-dose MTX) of ALL treatment. Interestingly, in the present study, the occurrence of relapse was found to be more in the third cycle of maintenance therapy. Intermittent discontinuation of chemotherapy or the repetitive adjustment of dose during the second cycle might have reduced the efficacy of MTX, resulting in higher relapse during the third cycle of maintenance. A study has shown that the degree of myelosuppression correlates with relapse risk [10]. Therefore, it would be better to adjust the dose of MTX based on its levels in the body rather than absolute neutrophil count.

RFS and OS were lower in patients with WBC count >50, 000 cells/mm^3 at the time of diagnosis ($p < 0.05$), similar to other studies [18, 20, 42, 43]. In co-regression model, -317GG genotype was associated with lower RFS and OS which is discordant with Dulucq et al. and Salazar et al. study reports. Dulucq et al. study reported AA genotype remained significant with event-free survival (EFS) (event is either relapse or death or induction failure) in multivariate regression model [18] whereas the later study did not find any association between -317A>G variant EFS [21]. WBC count was also significantly associated with lower RFS and OS in regression model similar to Dulucq et al. study [18] but dissimilar to Salazar et al. findings [21].

In spite of the small sample size, the present study is sufficiently powered to show a statistically significant difference in the risk of relapse (statistical power of the risk of association >85 %). However, a large multicentric study needs to be carried out to further strengthen our study findings.

Conclusion and future directions

The GG genotype of *DHFR*-317A>G variant is associated with increased risk of ALL relapse and lower overall survival in South Indian population. Both variants of *DHFR* (-317AA and -680CA) are found to be associated with severe leucopenia caused by MTX. The results of the present study could further be strengthened by measuring the MTX levels and DHFR mRNA levels to establish their role in predicting the outcome of MTX treatment. In addition, screening for other SNPs in *DHFR* gene will help us in understanding the complex genetic interactions and in identifying the risk haplotypes.

Compliance with Ethical Standards All procedures performed in the present study involving human participants were in accordance with the

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