Interleukin-23 Receptor Gene Variants in Acute Lymphoblastic Leukemia and Their Relation to Prognostic Factors

Mohammadrasul Zareinejad[1], Afshin Samiei[2], Behnaz Valibeigi[3], Tahereh Gholami[1], Soheila Zareifar[4], Zahra Amirghofran[1,5]*

[1]Department of Immunology, Shiraz University of Medical Sciences, Shiraz, [2]Department of Immunology, Hormozgan University of Medical Sciences, Bandar Abbas, [3]Department of Pathology, [4]Department of Pediatric Hematology and Oncology, [5]Auoimmune Disease Research Center, Medicinal and Natural Products Chemistry Research Center. Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Interleukin (IL)-23 has an important role in tumor immune regulation. **Objective:** To investigate the possible association of interleukin-23 receptor (IL23R) gene variants rs1884444, rs10889677 and rs11209026 with development of acute lymphoblastic leukemia (ALL). **Methods:** The IL23R variants were studied in 164 ALL patients and compared to 175 healthy controls by polymerase chain reaction-restriction fragment length polymorphism. The relationship between these variants and clinical and laboratory features of the patients and response to therapy were evaluated. **Results:** No significant differences in genotype and allele frequencies existed between patients and controls. The rs1884444TG genotype was significantly lower in patients who relapsed (24.2%) compared to those without relapse (55.9%, p=0.006). Fewer patients who relapsed had evidence of the G allele (P=0.034). The TG genotype was associated with a longer complete remission at1804±116 days compared to other genotypes (<1217 days, p=0.028), however this result was not significant in multivariate analysis. The rs10889677 AA genotype and A allele was associated with age (p<0.041) and platelet number (p=0.03) in precursor-B cell ALL (B-ALL) patients. Both occurred more frequently in patients aged 2-10 years (63.6% and 66%, respectively) and in those with platelets $>100\times10^3\mu L$ (68.4% and 52.4%, respectively). **Conclusion:** Our findings showed a lack of association of the studied polymorphisms with the risk of ALL. The influence of the rs1884444 polymorphism on relapse rate and association of rs10889677 AA genotype with favorable prognostic factors suggest the influence of the studied polymorphisms on ALL response to therapy and prognosis.

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*Corresopnding author: Dr. Zahra Amirghofran, Department of Immunology, Medicinal and Natural Products Chemistry Research Center. Shiraz University of Medical Sciences, Shiraz, Iran, Tel/Fax: (+) 98 7132351575, e-mail: amirghz@sums.ac.ir

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignancy characterized by overproduction of lymphoblasts. Although this disorder affects both children and adults, it is most common in children with a peak incidence between 2–5 years of age (1,2). In Fars Province, Southern Iran, this rate in children less than 15 years of age was reported as 32 cases per one million people in 2001 with a peak incidence in children under the age of 4 years (3).

Interleukin (IL)-23 is a cytokine that belongs to the IL-12 family of cytokines (4). This cytokine, which acts as a bridge between innate and adaptive immunity, is mainly secreted by B cells, dendritic cells and macrophages (5). IL-23 is a heterodimeric cytokine that consists of two subunits, p40 and p19. This cytokine plays a key role in differentiation of T cells into the T helper (h)17 subset, which are important cells in mediating inflammatory responses (6). The role of IL-23 in supporting the survival of $CD4^+$ Th17 cells and the pathogenic role of these cells as shown in multiple animal models of inflammatory and autoimmune diseases indicates the involvement of the IL-23/Th17/IL-17 axis in T cell-mediated diseases (6).

In recent years investigators have reported a dual role for IL-23 in tumor immunity. IL-23 has a pre-tumor function and promotes tumor growth by increasing proinflammatory cytokines such as IL-17 and IL-22, Matrix metalloproteinase (MMP) and increasing angiogenesis (7-10). In addition, IL-23 has decreased tumor infiltration of $CD8^+$ T cells into the transformed tissues (7). On the other hand, the anti-tumor role of IL-23 has been demonstrated in a variety of murine tumor models. Results showed increased $CD8^+$ T cell infiltration and prevention of the formation of experimental metastases (9).

There are various studies reported the role of IL-23 in leukemia. Cocco ***et al.*** showed the up-regulation of IL-23R in B-ALL cells, compared with normal early B lymphocytes (11). They observed that IL-23 could diminish directly leukemia cell growth *in vitro* and *in vivo* through the inhibition of cell proliferation and induction of apoptosis. This antileukemic activity of IL-23 was related to IL-23-induced up-regulation of miR15a expression resulted in down-regulation of B cell lymphoma (Bcl)-2 protein expression in B-ALL cells (11). The anti-leukemic activity of IL-23 through inducing peripheral blood mononuclear cells to express

Interferon (IFN)-γ, perforin and granzyme B have been shown by zhou ***et al.*** (12). Moreover, increased levels of IL-23 in peripheral blood and bone marrow mononuclear cells from newly diagnosed B-ALL patients compared with normal subjects have been demonstrated (13). These data suggest the involvement of IL-23 in the pathogenesis of leukemia and candidate IL-23 for the treatment of B-ALL patients unresponsive to standard therapy protocols (11).

Both the p40 and p19 subunits of IL-23 bind to a receptor complex comprised of the IL-23 receptor (IL23R) and the IL-12 receptor β1 (IL-12Rβ1) (14). IL23R is expressed on activated memory T cells, natural killer (NK) cells and at a lower level on monocytes, macrophages, and dendritic cells (14). Binding of IL-23 to IL23 results in tyrosine kinase2- and Janus kinase (JAK) 2-mediated signal transduction, along with activation of signal transducer and activators of transcription (STAT)3, STAT4 and STAT5 (14).

The IL23R gene is located on chromosome 1p31. A number of single nucleotide polymorphisms (SNPs) exist for this receptor, among which some are functional (15,16). Various studies have reported the association of IL23R polymorphisms with several autoimmune diseases such as rheumatoid arthritis, Inflammatory bowel disease

and Graves' ophthalmopathy (17-19). This cytokine's polymorphisms are associated with the development of gastric, esophageal and ovarian cancers (20-22).
We took into consideration the important role of the IL-23-IL23R interaction in tumor immunity in order to investigate the association of IL23R with susceptibility to ALL. For this purpose, the frequency of three functional common variants of IL23R-rs1884444 T>G, 10889677 A>C, and rs11209026 G>A were investigated in two groups, Iranian patients with ALL and healthy individuals. These three SNPs encode an amino acid change in the protein product and have functional consequences. In addition, previous studies have shown the importance of these variants in various cancers (20-22). The relationship between these variants to prognostic factors and response to therapy were also examined.

MATERIALS AND METHODS

Subjects. We recruited 164 patients with ALL and 175 healthy controls to participate in this study. Patients were hospitalized between 2011 to 2014 in ShahidFaghihi and Amir Oncology Hospitals, Shiraz, Iran. The diagnosis of ALL was confirmed according to pathological examination and clinical features reported by a relevant specialist. Patients ranged in age from 1-18 years (7.5 ± 4.09 (SD) years). Patients clinical and laboratory characteristics at presentation that included sex, age, white blood cell (WBC) counts, platelet counts, immunophenotype, hemoglobin (Hb) level, percentage of blast in the bone marrow and peripheral blood, and extramedullary involvement (EMI) of the central nervous system (CNS), liver, spleen and lymph nodes were collected from their medical documents during the time of hospitalization. During the follow-up period, we recorded the complete remission (CR) rate (the percentage of patients who obtained CR after initial therapy), complete remission duration (CRD) (the time calculated from the date of diagnosis, if relapsed to the first relapse date, otherwise to the recording date) and survival duration (the time calculated from the date of diagnosis to recorded date or date of death). Patients were treated according to the Children's Oncology Group (COG) protocol (23). Treatment was based on the patients' prognostic factors and leukemia type for total 2 to 3 years and involved administration of multidrug regimen in phases of remission induction, consolidation, prevention of CNS leukemia, and maintenance therapy. We divided the patients on the basis of prognostic factors and according to the protocol into two groups: i) standard [WBC:$<50\times10^3/\mu L$ and age 1 to <10 years] and ii) high-risk [WBC: $\geq50\times10^3/\mu L$ or age <1 and ≥10 years] (24). The control subjects were healthy individuals under the age of18 [mean: 7.5 ± 4.1 (SD)] years who visited Motahari Outpatient Clinic for regular examinations and had no evidence of familial malignancy or other serious disorders. Informed consent was given by the participants or their parents. The study protocol was approved by the Shiraz University Ethics Committee.
Patient Samples and Genomic DNA Extraction. Blood samples in EDTA-containing tubes were obtained from patients and controls. Genomic DNA was extracted from whole blood samples by Genetbio DNA blood Kit (South-Korea) according to the manufacture's procedure. Final preparation was quantified by measuring absorbance at 260 and 280 nm and then extracts were stored at-20°C until used.
Genotyping. To investigate the association between IL-23R gene polymorphisms and susceptibility to ALL, DNA samples were examined for rs1884444, 11209026 and

10889677 SNPs by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Sequences of the primers were as follows; rs1884444 (F5′-CTTCTTCCTCCCTAATCAAAGGTTCCCAT-3′, R5′-GCTATTACT GCATCCCATTGAATAGTGGC-3′); rs10889677: (F5′-ATCGTGAATGAGGAGTTGCC-3′, R5′-TGTGCCTGTATGTGTGACCA-3′); rs11209026 (F5′-AGTCACTCTGTGGCCTAAAGT AAAG-3′, R5′-AGATTTTTCTAGTAAACAACTGAAATGA-3′) (22,25,26). Each PCR amplication was prepared in a total reaction volume of 20 µL containing: 10 µL 2X TEMPase Hot Start Master Mix Blue (Ampliqon, Denmark), 0.5µM of each primer (Bioneer, South Korea), and5-40 ng genomic DNA. The PCR reactions were as follows: Initial denaturation at 95°C for 15 min and then 35 cycles (denaturation at 95°C for 30 s, annealing at different temperatures (63.5°C for rs1884444, 66°C for rs10889677 and 65˚C for rs11209026) for 30 s, extension at 72˚C for 30s) followed by 1 cycle at 72°C for 5 min. The PCR products size (rs1884444, 188bp; rs11209026, 350 bp; and rs10889677, 470 bp) were visualized on a 2% agarose gel stained by safe stain dye (CinnaGenn, Iran) to detect the quality of the amplication. The PCR products (10 µL) were digested with proper restriction endonucleases (rs11209026, 2 unit Hpy188I (Biolabs, UK); rs1884444, 2 unitHaeIII (BsuRI) (Fermentase, Lituania); rs10889677, 2 unitMnlI (Fermentase, Lituania), 2 µL 10xbuffer and 7.8 µL distillated water to achieve the final volume of 20 µL. After incubation at 37°C for overnight, digested products were separated by electrophoresis on 3.5% agarose gel (Invitrogen, UK) and the bands were visualized by an ultraviolet transilluminator (Upland, CA).

Statistical Analysis. Hardy–Weinberg equilibrium for all genotypes was evaluated by Arlequin ver 3.1 software. Genotypes and the distribution of alleles between patients and controls were tested with Pearson's chi-square ($\chi 2$) and Fisher's exact tests. Haplotype analysis was determined by Arliquine software and analyzed with Epi-Info 2002 (CDC, Atlanta, GA) software. Other statistical analyses were carried out using SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL, USA). The normality of the quantitative data distribution was assessed using the Kolmogorov–Smirnov test. We used the *t-test* and one-way ANOVA model for data with normal distribution and their nonparametric alternatives (Mann Whitney and Kruskal Wallis) for other parameters. We used the logistic regression test to evaluate genotype associations for relapsed and non-relapsed patients, and the odds ratio (OR) with 95% confidence intervals (CI). Survival and CRD were estimated by the Kaplan–Meier method. Univariate analysis was carried out using the log rank test and multivariate analysis by Cox's proportional hazards regression model. Data were expressed as mean ± standard error (SE) unless otherwise specified. P-values less than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics. Table 1 summarizes the ALL patients' clinical and biological characteristics. Of the 164 ALL cases, 116(70.7%) were male and 48(29.3%) were female. The control group comprised 175 healthy individuals, 121(69.1%) males and 54(30.9%) females. Due to a number of limitations and incomplete documentation, we were unable to document prognostic factor data for all patients. Most patients (76.7%) had precursor-B cell ALL (B-ALL) and 23.3% had T-ALL. The initial WBC count for patients was $32.4\pm5.46\times10^3/\mu L$ and the platelet count was $150.9\pm15.4\times10^3\mu L$. The Hb

level was 8.87±0.30 g/dL. The bone marrow blasts were 84.85±0.9% whereas the peripheral blood blasts were 47.7±4.6%. EMI was present in 58.9% of patients as follows: splenomegaly (54.8%); CNS involvement (25.8%); hepatomegaly (45.2%); and lymphadenopathy (14.5%). After diagnosis, 75.8% of patients entered remission during 27.6±5.0 days of chemotherapy. During the follow up, 34.2% of patients relapsed. CRD and survival duration were 824±66 days (median: 617) and 1114±66 days (median: 1131), respectively. The rate of patients who survived until the end of the study was 90.6% (death rate: 9.4%). We placed the patients according to *risk* classification into standard (65.5%) and high (34.5%) risk groups.

Table 1. Clinical and laboratory characteristics of acute lymphoblastic leukemia (ALL) patients.

Variables	Patients
Number	164
Age (years)	7.70 ± 0.49
Sex:	
Male	116 (70.7)
Female	48 (29.3)
Lineage:	
B	(76.7)
T	(23.3)
WBC ($\times 10^3/\mu L$)	32.4 ± 5.46
Platelet ($\times 10^3/\mu L$)	150.9 ± 15.4
Hb (g/dL)	8.87 ± 0.30
BM blasts (%)	47.75 ± 4.6
PB blasts (%)	84.85 ± 0.9
Patients with EMI	(58.9)
CR rate	(75.8)
CRD(days)	824 ± 66
Survival duration (days)	1114 ± 66
Survival rate	(90.6)

Data are represented as number, (%) or mean ± SE. WBC: White blood cells, Hb: Hemoglobin, BM: Bone marrow, PB: Peripheral blood, EMI: Extramedullary involvement, CR: Complete remission, CRD: Complete remission duration.

Genotypic and Allelic Distribution. Genotypes and allelic frequencies of IL23R rs1884444, rs10889677, and rs11209026 SNPs are shown in Table 2. The Hardy–Weinberg P-values in both ALL cases and controls in each SNP exceeded 0.05. On evaluation of genotype distribution for rs1884444, 50 (30.5%) had the TT genotype, 76 (46.3%) had the TG genotype, and 38 (23.2%) had the GG genotype. The frequencies of

these genotypes in the controls were as follows: TT in 55 (31.4%), TG in 79 (45.1%) and GG in 41 (23.4%) subjects. The allele frequencies were 53.7% for T and 46.3% for the G allele in patients compared to 54.0% for T and 46.0% for the G allele in controls. There was no significant difference in genotype and allele frequencies for this SNP between patients and controls.

Table 2. Genotype and allele frequencies of IL23R gene polymorphisms in acute lymphoblastic leukemia (ALL) patients.

IL-23R SNPs	Patients (n =164)	Controls (n =175)	P Value
rs1884444Genotypes			
TT	50 (30.5)	55 (31.4)	0.99
TG	76 (46.3)	79 (45.1)	
GG	38 (23.2)	41 (23.4)	
Alleles			
T	176 (53.7)	189 (54.0)	0.97
G	152 (46.3)	161 (46.0)	
rs10889677Genotypes			
AA	55 (33.5)	70 (40.0)	
AC	74 (45.1)	70 (40.0)	0.46
CC	35 (21.3)	35 (20.0)	
Alleles			
A	184 (56.1)	211 (60.28)	0.26
C	144 (43.9)	139 (39.7)	
rs11209026Genotypes			
AA	0	0	
AG	7 (4.3)	8 (4.6)	1.00
GG	157 (95.7)	167 (95.4)	
Alleles			
A	7 (2.1)	8 (2.2)	0.81
G	321 (97.9)	342 (97.8)	

SNP:, Single nucleotide polymorphism

The genotype frequencies for the rs10889677 SNP were as follows: AA in 55 (33.5%), AC in 74 (45.1%) and CC in 35 (21.3%) patients. In controls, these values were 70(40%) for AA, 70 (40%) for AC, and 35 (20%) for CC. The allele frequencies in patients were 56.1% for A and 43.9% for C. In normal controls, there were 60.28% who had the A allele and 39.7% with the C allele.

Table 3. The relationship between IL23R gene polymorphisms, laboratory and clinical features, and response to therapy in acute lymphoblastic leukemia (ALL) patients.

	rs1884444				rs10889677				rs11209026		
Variables	GG	TG	TT	P	CC	AC	AA	P	GG	AG	P
Age (years)											
< 2	4 (19)	10 (47.6)	7 (33.3)	0.3	7 (33.3)	10 (47.6)	4 (19)	0.1	21 (100)	0	0.5
2-10	17 (27)	31 (49.2)	15 (23.8)		10 (15.9)	32 (50.8)	21 (33.3)		59 (93.7)	4 (6.3)	
>10	10 (27)	12 (32.4)	15 (40.5)		8 (21.6)	12 (32.4)	17 (45.9)		35 (94.6)	2 (5.4)	
Sex											
Male	24 (21.6)	49 (44.1)	38 (34.2)	0.3	24 (21.6)	50 (45)	37 (33.3)	0.5	105 (94.6)	6 (5.4)	0.6
Female	11 (25)	23 (52.3)	10 (22.7)		8 (18.2)	24 (54.5)	12 (27.3)		43 (97.7)	1 (2.3)	
WBC($\times 10^3 \mu L$)											
<10	12 (24)	20 (40)	18 (36)		10 (20)	22 (44)	18 (36)		46 (53.5)	3 (75)	
10-49	6 (25)	11 (45.8)	7 (29.2)	0.9	5 (20.8)	10 (41.7)	9 (37.5)	0.8	24 (27.9)	0	0.6
≥50	5 (29.4)	8 (47.1)	4 (23.5)		5 (29.4)	5 (29.4)	7 (41.2)		16 (18.6)	1 (25)	
Plt ($\times 10^3/\mu L$)											
<20	2 (12.5)	8 (50)	6 (37.5)		6 (37.5)	6 (37.5)	4 (25)		15 (93.8)	1 (6.3)	
20-99	6 (19.4)	16 (51.6)	9 (29)	0.4	6 (19.4)	13 (41.9)	12 (38.7)	0.4	30 (96.8)	1 (3.2)	1.0
≥100	13 (31)	15 (35.7)	14 (33.3)		7 (16.7)	17 (40.5)	18 (42.9)		40 (95.2)	2 (4.8)	
Hb(g/dL)											
<7	1 (4.5)	8 (21.1)	6 (20.7)	0.3	7 (35)	4 (10.8)	5 (15.6)	0.1	16 (18.4)	1 (25)	0.8
7-11	18 (81.8)	25 (65.8)	17 (58.6)		10 (50)	25 (67.6)	23 (71.9)		56 (64.4)	3 (75)	
>11	3 (13.6)	5 (13.2)	6 (20.7)		3 (15)	8 (21.6)	4 (12.5)		15 (17.2)	0 (0)	
PB blasts											
< 20	3 (20)	8 (53)	4 (26.7)	0.2	1 (6.7)	8 (53.3)	6 (40)	0.4	14 (93.3)	1 (6.7)	0.7
20-50	2 (16.7)	9 (75)	1 (83)		3 (25)	3 (25)	6 (50)		12 (100)	0	
>50	10 (40)	10 (40)	5 (20)		4 (16)	7 (28)	18 (34.6)		23 (92)	2 (8)	
BM blasts											
<50%	0	1 (100)	0	1.0	0	0	1 (100)	0.5	1 (100)	0	1.0
≥50%	20 (27.4)	30 (41.1)	23 (31.5)		16 (21.6)	31 (42.5)	26 (35.6)		69 (94.5)	4 (5.5)	
Lineage											
B-cell	13 (23.2)	24 (42.9)	19 (33.9)	0.4	14 (25)	25 (44.6)	17 (30.4)	0.2	54 (96.4)	2 (3.6)	0.2
T-cell	6 (35.3)	8 (47.1)	3 (17.6)		1 (5.9)	8 (47.1)	8 (47.1)		15 (88.2)	2 (11.8)	
CRD(days)	798±149	930.8±101	734±120	0.3	752±130	927±105	818±122	0.7	836±69	1087±354	0.5
Survival duration	1292±180	1062±93	1058±107	0.6	924±127	1195±113	1126±108	0.4	1089±61	1847±938	0.5
Risk groups											
Standard	12 (24.5)	22 (44.9)	15 (30.6)	0.9	9 (18.4)	23 (46.9)	17 (34.7)		46 (93.9)	3 (6.1)	0.6
High	13 (26)	20 (40)	17 (34)		12 (24)	19 (38)	19 (38)	0.6	48 (96)	2 (4)	

With respect to the rs11209026 SNP, none of the patients had the AA genotype, whereas 7 (4.3%) had the AG and 157 (95.7%) had the GG genotype. Corresponding data for controls was 0% for AA, 8 (4.6%) for AG and 167 (95.4%) for the GG genotype. The allele frequencies were 2.1% for A and 97.9% for the G allele in patients. In controls, these values were 2.2% for A and 97.8% for the G allele. Statistical analysis revealed no significant differences in genotypic distribution and allelic frequencies of rs10889677 and rs11209026 SNPs between the patients and controls ($p>0.05$).

A comparison of the IL23R haplotype frequencies of patients and controls showed no significant differences.

Genotype Distribution and Allele Frequencies in Relation to Patient Characteristics and Prognostic Factors. We assessed the relationship between each SNP and prognostic factors. There was no significant difference in the quantitative prognostic factors of age, WBC, platelets, Hb level, percentages of blasts in the bone marrow and the peripheral blood between the different genotypes. There was no significant difference between the frequencies of various genotypes in T-ALL and B-ALL patients (Table 3).

The patients were categorized according to age, WBC counts, platelet counts, Hb level, percentage of blastocytes in the bone marrow and peripheral blood (Table 3). We compared the frequency of genotypes and alleles in each group. No significant difference existed between genotype distribution in the different categories except for age when patients were categorized into the following age groups: <2 years, 2-10 years and >10 years, and platelet counts in B-ALL patients (Table 4).

Table 4. Association of the IL23R rs10889677 variant with age and platelet groups in precursor-B cell acute lymphoblastic leukemia (B-ALL) patients.

rs10889677	Age (years)			P Value
	<2	2-10	>10	
Genotypes				
AA	1 (4.5)	14 (63.6)	7 (31.8)	
AC	7 (22.6)	21 (67.7)	3 (9.7)	0.015
CC	5 (31.3)	5 (31.3)	6 (37.5)	
Alleles				
A Present	8 (15.1)	35 (66.0)	10 (18.9)	0.041
Absent	5 (31.3)	5 (31.3)	6 (37.5)	
C Present	12 (25.5)	26 (55.3)	9 (19.1)	0.08
Absent	1 (4.5)	14 (63.6)	7 (31.8)	
rs10889677	Platelet number ($\times 10^3/\mu L$)			P Value
	<20	20-99	>100	
Genotypes				
AA	2 (10.5)	4 (21.1)	13 (68.4)	
AC	3 (13.0)	11 (47.8)	9 (39.1)	0.03
CC	6 (46.2)	3 (23.1)	4 (30.8)	
Alleles				
A Present	5 (11.9)	15 (35.7)	22 (52.4)	0.03
Absent	6 (46.2)	3 (23.1)	4 (30.8)	
C Present	9 (25.0)	14 (38.9)	13 (36.1)	0.09
Absent	2 (10.5)	4 (21.1)	13 (68.4)	

Data are represented as frequency or (percentage). Fisher exact test was used to analyze the data.

In this group of patients, the frequency of the rs10889677 AA genotype was 4.5% in patients <2 years; 63.6% in patients 2-10 years and 31.8% in patients >10 years (p=0.015) with an increased AA genotype in the 2-10 year age group. Accordingly, the frequency of the A allele in patients 2-10 years (66.0%) of age was more than two other age groups (OR: 4.2, 95% CI: 1.05-16.6, p=0.041). With respect to the association between rs10889677 genotypes and platelet numbers in B-ALL patients, there was a higher frequency of the AA genotype in patients with platelets>100×10^3µL (68.4%) compared to those with platelets<20×10^3µL (10.5%) and in those with 20-99×10^3µL (21.1%) platelets (p=0.03). The A allele was higher in patients with >100×10^3µL (52.4%) versus patients with<20×10^3µL (11.9%) and those with 20-99×10^3µL (35.7%) platelets (OR: 6.6, 95% CI: 1.3-33.5, p=0.03; Table 4). A significant association existed between age groups and platelet counts (p=0.001), WBC counts (p=0.022) and Hb level (p=0.016) in B-ALL patients. There were 80.8% of children with>100×10^3µL platelets, 65% of patients with Hb levels 7-11 g/dL and 73.0%of patients with<10×10^3µL WBCs in the 2-10 year age group.

None of the genotypes showed an association with the occurrence of EMI. There were also no significant differences in the frequencies of different alleles and genotypes in the two risk groups (Table 3).

Genotype Distribution and Allele Frequencies in Relation to Response to Therapy. Table 3 shows CRD and survival duration of patients in relation to genotype.

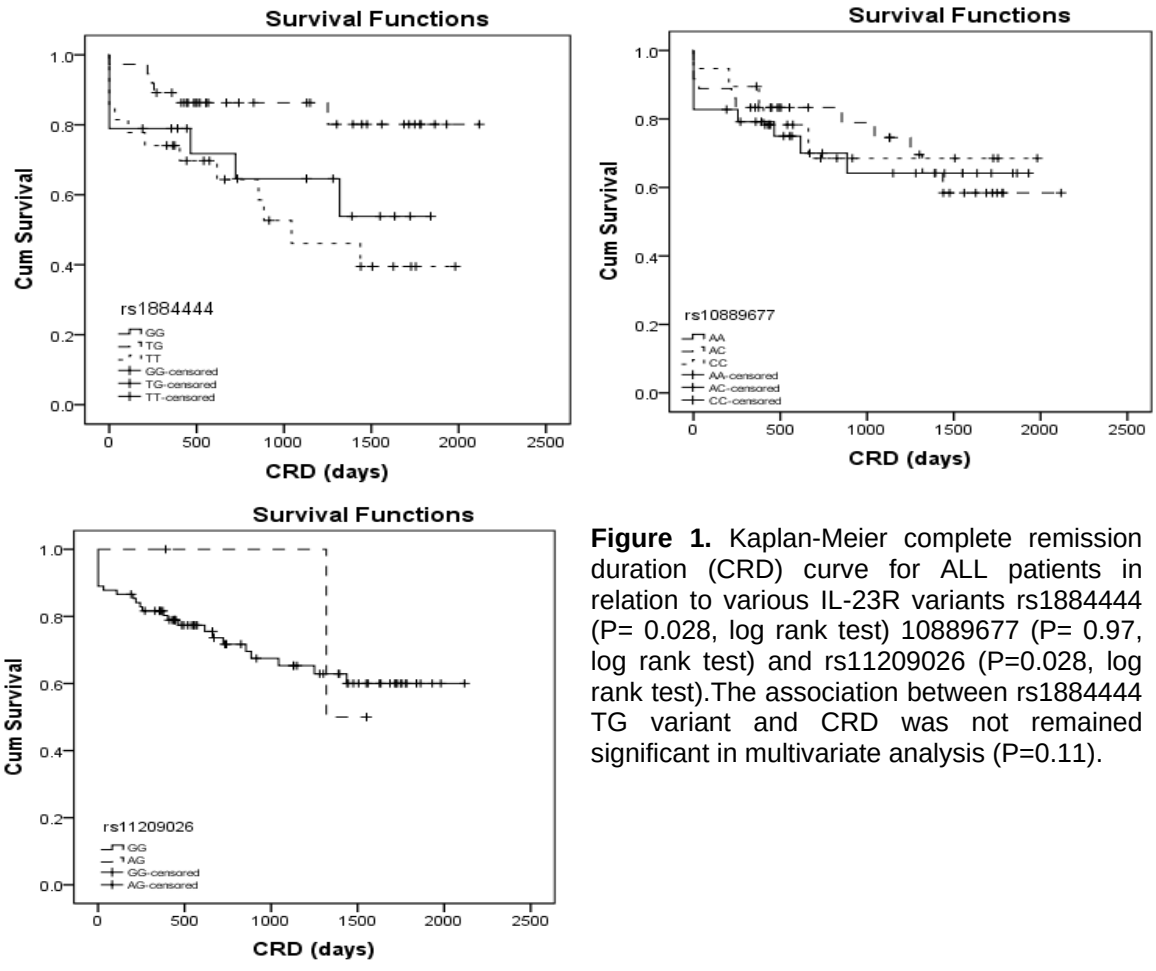

Figure 1. Kaplan-Meier complete remission duration (CRD) curve for ALL patients in relation to various IL-23R variants rs1884444 (P= 0.028, log rank test) 10889677 (P= 0.97, log rank test) and rs11209026 (P=0.028, log rank test).The association between rs1884444 TG variant and CRD was not remained significant in multivariate analysis (P=0.11).

We used the time-to-event analysis by Kaplan–Meier method to estimate the overall survival and CRD of patients with different genotypes. No significant difference except for CRD in relation to the rs1884444 variant was found. As seen in Figure 1, patients with rs1884444 TG genotype had a longer CRD (1804 ± 116 days) compared to patients with the GG (1217 ± 186 days) and TT (1110 ± 168 days) genotypes (hazard ratio (HR):0.29, 95% CI: 0.11-0.77, p=0.013).This association did not remain significant in multivariate analysis (HR:0.08, 95% CI: 0.004-1.7, p=0.11) after adjustments for age, immunophenotype, platelet and WBC number, EMI and the percentage of blastocytes in bone marrow and peripheral blood.

There was an association between the rs1884444 TG genotype and G allele with rate of relapse. This genotype was significantly lower in patients who relapsed (24.2%) compared to those without relapse (55.9%). Logistic regression analysis of the effect and relation of genotypes on relapse showed a significant association between the TG genotype and risk of relapse (OR: 0.23, 95% CI: 0.08-0.66, p=0.006). In TG patients, there was 77% less relapse compared to patients with the TT genotype, which showed the protective effect of the TG genotype in ALL patients. The GG genotype had a nonsignificant protective effect compared with TT patients (OR: 0.62, 95% CI: 0.21-1.8, p=0.39). When allele association analysis was performed, there were fewer G allele patients who relapsed (OR: 0.37, 95% CI: 0.15-0.92, p=0.034).

DISCUSSION

ALL is a type of blood cancer that progresses rapidly and creates immature blood cells (27). A number of studies have investigated the importance of different molecules in thedevelopment and prognosis of ALL (28-30), including the important role of the IL-12 cytokine family and their immunological and anti-tumor functions in the control of pediatrics ALL (11). IL-23 is a proinflammatory cytokine that belongs to this family. Although the anti-tumor activity of IL-23 is controversial, functional experiments have shown that IL-23 can diminish leukemia cell growth both *in vitro* and *in vivo* through suppression of cell proliferation (11). IL-23 can induce apoptosis in leukemia cells through up-regulation of miR15a expression which results in down-regulation of the B cell lymphoma (Bcl-2) anti-apoptotic molecule(11).

We took into consideration the importance of IL-23 and its receptor in the biology of ALL. To the best of our knowledge, no previous study has investigated the role of IL23R variants in susceptibility to ALL. Therefore, this study examined the association of three functional IL23R SNPs and the risk for ALL development.

The non-synonymous SNP rs1884444 T>G is located on exon 2 of IL23R.This exon encodes the signal peptide of IL23R (31). Substitution of amino acid His by Gln in this signal sequence at the extracellular domain of IL23R may influence its specificity and affinity to IL-23 (22), and thus modulate the Th17 cell-mediated immune response which is involved in autoimmune diseases and cancer pathogenesis (6).

In a previous study, Xu *et al.* found an association between the GG genotype of rs1884444 and susceptibility to hepatocellular carcinoma (32). The association between the rs1884444 G allele with a decreased risk of gastric cancer has also been reported by Chen *et al.*(22). With respect to hematological malignancies, Qian *et al.* observed a significant association between the rs1884444 SNP and susceptibility to acute myeloblastic leukemia (AML) in a Chinese population. In their study, the TG genotype

and the TG/GG combined genotypes was associated with an increased risk of AML (33). In the current study, there was no significant difference in rs1884444 genotypes and alleles between patients and controls, which indicated the lack of an association between this polymorphism and susceptibility to ALL. A study of the relationship of this SNP to patient characteristics showed no significant association with different prognostic factors. However we found an association between the rs1884444 TG genotype and longer CRD in patients. This association did not remain significant in multivariate analysis which indicated that this genotype in combination with other prognostic markers might have not an impact on therapeutic response. This genotype was less frequent in patients who relapsed compared with those without relapse. The G allele was also significantly lower in patients who relapsed. These findings might imply the influence of this IL23R variant on decreased risk of relapse in patients and suggested the importance of the rs1884444 TG genotype in relation to patient response to therapy. In a previous study by Wu *et al.,* a decreased Th17 cell frequency in AML patients who had achieved CR after chemotherapy has been reported (34). IL-17A secreted by Th17 cells, has induced the proliferation of AML cells via IL-17R, in which activation of downstream kinases (PI3 kinase and Akt) and JAK/STAT3 signaling pathway may play significant roles (34). In addition, combination of IL-17 and IL-22 significantly decreased deviation of T cells toward Th1 cells and IFN-γ secretion from peripheral blood mononuclear cells of AML patient (34). AML patients with high Th17 cell frequency have shown poor prognosis (34). Bi *et al.* showed that Th17-secreted cytokines could induce the proliferation of B-ALL cell line Nalm-6 and B-ALL cells isolated from the patients by phosphorylation of Akt and STAT3. The increased Th17 cells and IL-17 existed in the B-ALL patients promoted proliferation and resistance to daunorubicin treatment via activation of Akt signaling (13). These data shows that the IL-23/Th17 axis may contribute to the prognosis and response to therapy in ALL patients and IL-23 may have an important role in ALL therapy by modulating the expression of Th17 cells-associated molecules. As mentioned above, the presence of rs1884444 G allele instead of T allele may reduce affinity or specificity of IL-23R resulting in decreased levels of IL23R signaling and diminished generation of Th17 responses. Whether reduced Th17 levels were the main reasons for the decreased rate of relapse observed in ALL patients with the TG genotype would warrant further investigation.

The second SNP examined in this study (rs10889677*A*>C) is located in the 3'-UTR and may cause overexpression of IL23R by increasing mRNA stability and the driving differentiation of T cells towards a Th17 subpopulation (25). This, in turn, may contribute to tumorigenesis by promoting angiogenesis and inducing MMP through IL-17 release (6). This SNP may influence T-cell proliferation, resulting in changes in the levels of regulatory T cells and modifications to cancer susceptibility (35). Zhang *et al.* have demonstrated a significantly higher frequency of the rs10889677 C allele in patients with ovarian cancer compared with controls (21). The same results have been reported by Chien *et al.* for allele C in oral cancer patients. In these patients, this allele was associated with a higher risk of lymph node metastasis (36). In our study, we found no association of this SNP with either susceptibility to ALL, or with various prognostic factors in total patients. However, there was a significant association in B-ALL patients between this SNP and patient age group. As our data showed, a significantly higher frequency of the AA genotype existed in patients 2-10 years of age. In various studies this age group showed better responses to treatment (24,37). In the current study,

patients in this age range had lower WBC and higher platelet counts which favored a good prognosis. Therefore, the higher frequency of AA genotype and A allele in patients 2-10 years of age might imply the importance of this genotype as a good prognostic factor. This result was supported by our other finding in which the AA genotype was more frequent in patients with platelets $>100\times10^3/\mu L$.

The third SNP, non-synonymous rs11209026 G>A (Arg381Gln), is located in the JAK2 kinase-binding domain of IL23R. It is possible that the change from Arg to Gln results in attenuated IL-23 signaling (38). In a recent study, the IL23R rs11209026 A allele and AA genotype have shown no correlation with AML susceptibility (12). A number of studies found a significant association between rs11209026 and susceptibility to various autoimmune diseases (19,39). In limited studies performed on cancer patients, this variant did not show a significant association with colorectal cancer, however there was a protective role in hepatitis C related hepatocellular carcinoma (40,41). In the current study, due to low frequency of A allele none of the patients had AA genotype. The same finding has been recently reported by Nemati *et al.* on Iranian subjects (40). There was no association of this SNP with susceptibility to ALL, various prognostic factors, and response to therapy.

This study has several limitations that include relatively low numbers of patients and controls which were mostly due to the difficulty of obtaining samples from children. In a number of patients, we were unable to obtain all clinical and laboratory information, which included cytogenetics data, due to the incompleteness of their medical records.

In conclusion, we found no significant difference in genotype and allele frequencies of the three studied IL23R SNPs between the patient and control groups. This finding has implied a lack of association of these variants with the risk of ALL. Hence, additional studies with higher numbers of patients that include an investigation of other SNPs are recommended. With respect to the relationship to prognostic factors and therapy response, we have found an association between the rs1884444 TG genotype and G allele with a lower rate of relapse. Nevertheless, this genotype was not confirmed to be an independent variable that affected CRD. There were significantly more B-ALL patients with the 10889677 AA genotype and A allele who had a favorable age range and platelet counts which might suggest a possible contribution of this genotype and allele to ALL prognosis.

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