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RESEARCH

Fas Gene Variants in Childhood Acute Lymphoblastic Leukemia and Association with Prognosis

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Abstract Fas molecule is one of the main important molecules involved in apoptotic cell death. Single nucleotide polymorphisms in the promoter of Fas gene at positions −1377G/A and −670 A/G may affect its expression and play an important role in the pathology of leukemia. In the present study the association between these polymorphisms and risk of the development of acute lymphoblastic leukemia (ALL) in children with ALL compared to cancer-free control subjects was examined by polymerase chain reaction- based restriction fragment length polymorphism. The relationship between the polymorphisms and clinical and laboratory features of the patients and response to therapy were determined. No significant differences in genotype and allele frequencies between the patients and the control subjects at positions −670 and −1377 were detected. Evaluation of the prognostic factors revealed an association between the GG genotype at position −670 and liver involvement in ALL patients ($p < 0.04$). Although patients with −1377 AA genotype showed shorter mean complete remission duration, the result of survival analysis did not reach to be significant. In conclusion, results of this study showed no contribution of Fas genotypes at positions −670 and −1377 to risk of ALL in children. The association of Fas GG genotype at position −670 with liver involvement in the patients may show its important role in prognosis of ALL.

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Introduction

Programmed cell death, or apoptosis is a biological process that plays an essential role in development and homeostasis of the hematopoietic system [1]. Several evidences have suggested that dysregulation of this pathway result in development of a variety of human diseases and persistence of malignant clones by reducing the death rate of cells [2]. Fas also known as TNFSF6/CD95/APO-1, belongs to the superfamily of tumor necrosis factor receptors and is one of the essential molecules contributing in extrinsic pathway of apoptosis in many cell types [3–5]. The human Fas gene located on chromosome 10q 24.1 [6], is composed of nine exons and eight introns [7]. Mutations or polymorphisms might influence Fas gene expression [8]. Two functional single nucleotide polymorphisms (SNPs) in the promoter region of this gene have been reported [9]. One of these polymorphisms is A to G substitution at position −670 in the enhancer region that modulates signal transducer and activator of transcription (STAT-1). Although the direct effect of STAT1 on Fas expression has not yet been documented, the G allele has been suggested to contribute to Fas dysregulation [10]. The other polymorphism is G to A base change at position −1377 situated between two putative silencer regions that results in destroying stimulatory protein (SP1) [9]. This polymorphism has shown to diminish promoter activity and Fas expression level by decreasing transcription factors binding [9]. Reduced expression of Fas results in down regulation of Fas-mediated apoptosis that is associated with an increased risk of cancers [11]. In various studies the correlation between Fas polymorphisms and pathogenesis of malignancies such as some solid cancers including carcinoma

of the head and neck [12], nasopharynx [13], prostate [14] intestine [15] and lung [16] has been shown. Association of this gene and hematological malignancies including Hodgkin's lymphoma [17, 18], multiple myeloma [19], adult T-cell leukemia (ATL) [20], and adult acute myeloid leukemia (AML) [9] have also been investigated. In some of these studies, the association between Fas gene polymorphisms with disease susceptibility and with different prognostic factors has been shown. To our knowledge, the influence of the above Fas gene polymorphisms on the susceptibility to acute lymphoblastic leukemia (ALL) in children and their relation to prognostic factors has not yet been studied. In the present work we deal with this issue to more understand the impact of genetic Fas variants on development and outcome of ALL.

Materials and Methods

Subjects

A total of 142 patients who were hospitalized for ALL in Faghihi and Nemazi hospitals (Shiraz, south of Iran) during years 2008–2011were entered the study. Of total 85 patients (59.9 %) were male and 57 (40.1 %) were female, with a mean age of 7.3±4.4 years (range, 1–17 years). The patient's disease was confirmed as ALL according to pathological and clinical features. Clinical and laboratory characteristics of the patients at presentation including sex, age, French American British (FAB) morphological classification, white blood cell (WBC) and platelet numbers, hemoglobin (Hb) level, percentage of blast in bone marrow and peripheral blood, extramedullary involvement (EMI) of central nervous system (CNS), liver, lymph node, spleen and testis, and cytogenetics [t(12;21), t(1;19), t(9;22), t(4;11) and hyperdiploidy] were recorded from their medical documents during the time of hospitalization. All patients were treated according to the ALL IC-BFM 2002 protocol. During the follow-up, the complete remission (CR) rate, complete remission duration (CRD) and survival duration were recorded. The patients were divided into three risk-groups of standard-risk (SR), intermediate-risk (IR) and high-risk (HR) based on age, initial WBC count at diagnosis and percentage of blasts in bone marrow on day 15 of treatment. We defined the SR group by age 1–6 years and WBC $< 20\times10^9$/L and BM blasts <5 % on day 15, IR by age ≥6 years or WBC $\geq20\times10^9$/L, BM blasts <5 % on day 15 and HR by the presence of t(9;22) and t(4;11) fusions or 5–25 % or more than 25 % blasts in bone marrow on day 33. During the study period those patients who were admitted to the hospital due to relapse were also included in the study. Control group consisted of 117 cancer- free subjects, 76 (65 %) males and 41 (35 %) females, having a mean age of 8.8±4.05 years, ranged from 1 to17, with no evidence of familial malignancy or autoimmune disorders. Patients and control subjects did not significantly differ regarding age and sex ($p=0.3$). The protocol of the study was approved by the Ethics Committee of Shiraz University of Medical Sciences. All subjects agreed to participate in this study and informed consent was obtained from them or their parents.

DNA Isolation

A total of 2 ml blood was obtained from the patients and controls in EDTA-containing tube. Mononuclear cells were separated using Ficoll-hypaque gradient centrifugation. Genomic DNA was extracted from all subjects using QIAamp, DNA blood Mini kit (QIAgen, Hilden, Germany) according to the manufacture's protocol and then stored at −20 °C until used.

Primers Selection and PCR Amplification

Genotypes were identified by polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP). Two different reaction mixtures were provided for each sample for the detection of Fas promoter region at positions −670 and −1377 in patients and controls. PCR amplification was prepared in a total volume of 25 μl containing: 100 ng of genomic DNA, 2.5 μl of reaction buffer 10×, 200 μM of deoxyribonucleotide triphosphate (dNTP), 1.5 mM of $MgCl_2$, 1 unit of Taq DNA polymerase (Cinnagenn, Tehran, Iran) and 0.4 μM of each primer (TIB MOLBIOL, Berlin, Germany). Primer sequences designed according to the previous studies [21] are shown in Table 1. PCR mix without DNA was used as a negative control to ensure the contamination-free PCR product. PCR program was run at 94 °C for 10 min, followed by 35 cycles of (94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min) and final extension at 72 °C for 5 min. Amplification was confirmed by electrophoresis on 2 % agarose gel. PCR products (10 μl) were digested with 1 unit of MvaI and 10 units of BstUI restriction endonucleases (Fermentas, Lituania) for −670A/G and −1377G/A polymorphisms, respectively. The mixture was incubated at 37 °C for overnight, then separated on 2.5 % agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination (Gel Doc 2000, Italy). BstUI generated one fragment of 122-bp from A allele, two fragments of 103-base pair (bp) and 19-bp from G allele at position −1377 (Table 1). MvaI generated a 232-bp fragment from A allele and 184, 101 and 47-bp fragments from G allele at position −670 (Fig. 1).

Table 1 Primer sequences and DNA fragments generated by restriction enzymes for Fas gene polymorphisms by PCR-RFLP

Positions	Primers	Length (bp)	Restriction enzyme	Fragments size (bp)
−1377 G/A	F 5′-TGTGTGCACAAGGCTGGCGC-3′	122	Bsh1236I (BstUI)	GG: 103,19
	R 5′-TGCATCTGTCACTGCACTTACCACCA-3′			GA: 122, 103,19
				AA: 122
−670 A/G	F 5′-CTACCTAAGAGCTATCTACCGTTC-3′	332	MvaI (BstNI)	AA: 232,101
	R 5′-GGCTGTCCATGTTGTGGCTGC-3′			AG: 232, 184, 101,47
				GG: 184, 101, 47

Statistical Analysis

Data analysis was carried out using SPSS 16 (SPSS Inc, Chicago, IL, USA) and Epi-Info 2002 (CDC, Atlanta, GA). Hardy-Weinberg equilibrium, demographic variable distribution, genotype and allele frequencies between patients and controls were analyzed with Pearson chi-square (χ^2) statistic test. The relationship between Fas gene polymorphisms and prognostic factors was determined by chi-square and Fisher's exact tests. CR rate was defined as the percentage of patients who obtained complete remission after initial therapy. CRD was evaluated from the date CR was achieved. Duration of survival was calculated from the date of diagnosis. Survival rate was determined as percentage of patients who were alive after diagnosis or treatment till the end of study. Overall survival and CRD were estimated using the Kaplan-Meier method. Univariate analysis was performed using the log rank test and multivariate analysis by Cox's proportional hazards regression. A predictive value of less than 0.05 was used as the statistical significance.

Results

Characteristics of the Study Population

Clinical and biological characteristics of the patients are summarized in Table 2. Both case and control groups were in Hardy-Weinberg equilibrium at positions −670 and −1377. The majority of patients (90.7 %) had precursor-B cell ALL (B-ALL) and 9.3 % had T-ALL. The mean WBC and platelet count of the patients at diagnosis were $38.8\pm62.1\times10^9$/L and $94.7\pm100\times10^9$/L, respectively. EMI was present in 65.7 % of cases. Due to some limitations cytogenetic data of only 52 patients were available of which 38.5 % had abnormal cytogenetic. 84 of 109 patients that their clinical data were available entered remission (CR rate, 77.1 %). The mean survival and CR durations were 1288±537 days (median, 1022) and 968±510 days (median, 952), respectively.

Fas Promoter Polymorphisms at Positions −670 and −1377

Genotype and allele frequencies of −670 A/G and −1377G/A Fas gene polymorphisms in ALL patients and controls are

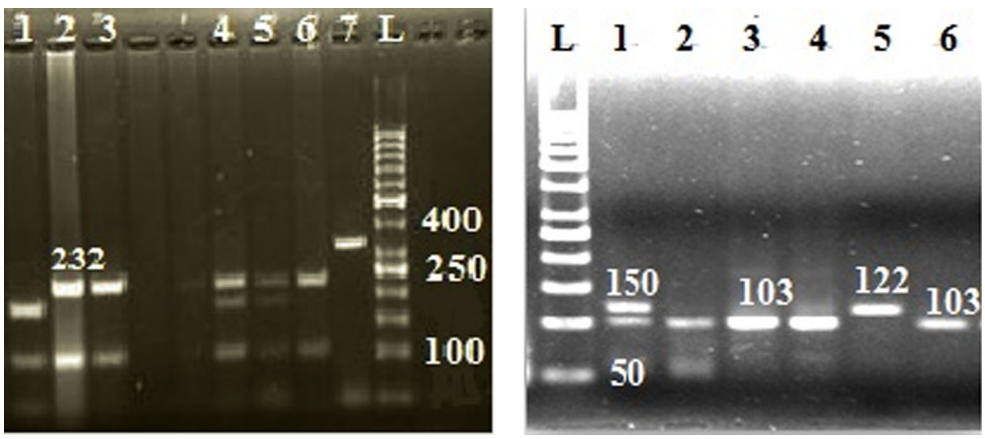

Fig. 1 PCR-RFLP results of −670 A→G substitution in the Fas promoter region. Lane 1: GG homozygous. Lane 2, 3 and 6: AA homozygous. Lane 4 and 5: AG heterozygous. Lane 7: PCR product of −670 region. Right: PCR-RFLP results of −1377 G→A substitution in the Fas promoter region. Lane 1: GA heterozygous. Lane 2, 3, 4 and 6: GG homozygous. Lane 5: AA homozygous and PCR product of −1377 region. Molecular weight marker is 50 bp (Fermentas, Lithuania)

Table 2 Characteristics of patients with ALL

Variables	Total
Patients	142
Age	7.3±4.4
Range (year)	1–17
1–6 years	73 (51)
≥ 6 years	69 (49)
Male/Female	85 (59.9)/57 (40.1)
WBC $\times 10^9$/L	38.8±62.1
Platelet $\times 10^9$/L	94.7±100
Hb (g/dl)	8.5±4.4
Blast in PB	(50.2±31.4)
Blast in BM	(72.6±24.3)
Lineage: B/T	(90.7)/(9.3)
Abnormal cytogenetics	20 (38.5)
Patients with EMI	67 (65.7)
CRD (days)	968±510
CR rate	(77.1)
Survival duration (days)	1288±537
Survival rate	(98)

All parameters are in number (percentage). Values for age, white blood cell (WBC), platelet, hemoglobin (Hb), complete remission duration (CRD) and survival duration are mean±SD. *EMI* extramedullary involvement, *CR* complete remission. *PB* peripheral blood. *BM* bone marrow

shown in Table 3. As indicated, at position −670, out of 142 ALL patients, 31 % AA, 14.7 % GG and 54.3 % had AG genotypes. Among the controls 40.2 % AA, 11.1 % GG and 48.7 % had AG genotypes. On evaluation of genotype distributions at −1377 position, 2.8 % of patients had AA genotype, 82.4 % had GG and 14.8 % had GA genotypes. Statistical analysis revealed no significant differences in genotype frequencies at −1377 bp between ALL patients and controls, also when the patients were categorized according to sex ($P=0.78$). There were no significant difference in allele frequencies between patients and healthy individuals at both positions. On evolution of the allele and genotype distributions between B-lineage ALL (B-ALL) patients and controls at positions −670 and −1377, no significant differences was found (data not shown).

Table 3 Genotype and allele frequencies of −670 A/G and −1377G/A Fas gene polymorphisms in ALL patients

Position		Patients ($n=142$)	Controls ($n=117$)	P-value
−670	Genotype			
	AA	44(31)	47(40.2)	0.27
	AG	77(54.3)	57(48.7)	
	GG	21(14.7)	13(11.1)	
	Allele			
	A	165(58.1)	151(64.5)	0.13
	G	119(41.9)	83(35.5)	
−1377	Genotype			
	GG	117(82.4)	94(80.3)	0.63
	GA	21(14.8)	17(14.5)	
	AA	4(2.8)	6(5.2)	
	Allele			
	G	255(89.8)	205(87.6)	0.43
	A	29(10.2)	29(12.4)	

Data are presented as number (%)

Relationship between Fas Genotypes and Prognostic Factors

The impact of each genotype at positions −670 and −1377 on established prognostic factors was analyzed and the results are presented in Table 4. No significant association of −670 genotypes with age, WBC and platelet numbers, peripheral blood and bone marrow blast percentages, cytogenetics and FAB subtypes in ALL patients was observed. There was also no significant difference in genotypes between patients presented with EMI and those without, however a correlation between −670 polymorphism and the presence of hepatomegaly in the patients was found ($p<0.04$, Table 5). GG genotype was observed in 8.3 % of patients with liver involvement compared to 91.7 % in those without liver involvement. This lower frequency of −670 GG genotype in hepatomegaly-positive patients than-negative ones suggests the association of this genotype with a better prognosis in ALL patients. There was no significant relationship between the above prognostic factors and Fas gene polymorphism at −1377 position.

In this study, the influence of the polymorphisms on response to therapy in patients was also examined. Since 98 % of patients were alive at the end of study, analysis of overall survival in relation to different genotype variants was not performed. There were no remarkable differences between CRD and CR rate in different Fas genotypes at −670 positions (Table 4). With respect to −1377 genotypes, patients with the variant AA had a shorter mean CRD compared to other genotypes, however, according to univariate and multivariate analysis this genotype did not reach a statistical significance (log rank test, $p=0.07$). Survival curves for the patients in relation to −670 and −1377 variants estimated by the Kaplan-Meier method are shown in Fig. 2. The frequencies of risk groups were 21.1 % for SR group, 60.6 % for IR group and 18.3 % for HR group. Statistical analysis also indicated no significant differences between the frequency of Fas genotypes (at −670 and −1377) and three risk-groups (Table 4).

Table 4 The relationship between Fas gene promoter polymorphisms at −670 and −1377 positions and laboratory and clinical features of the ALL patients

	−670				−1377			
Variables	GG	AA	AG	*P* value	GG	AA	GA	*P* value
Total No.	21(14.7)	44(31)	77(54.3)		117(82.4)	4(2.8)	21(14.8)	
Age								
1–6 years	11(15)	20(27.4)	42(57.6)	0.62	60(82)	2(2.7)	11(15.3)	0.99
≥6 years	10(14.5)	24(34.7)	35(50.8)		57(82.6)	2(2.9)	10(14.5)	
Male	12(14.1)	25(29.4)	48(56.5)	0.8	68(80)	2(2.4)	15(17.6)	0.47
Female	9 (15.8)	19 (33.3)	29(50.9)		49(86)	2(3.5)	6(10.5)	
WBC $\times 10^9$/L								
< 20	13(18)	24(33)	35(49)	0.15	60(83.3)	3(4.2)	9(12.5)	0.58
≥ 20	6(38.3)	3(7)	23(54.7)		33(78.5)	1(2.3)	8(19.2)	
Platelet $\times 10^9$/L								
< 100	15(19)	20(25)	45(56)	0.41	64(80)	4(5)	12(15)	0.37
≥ 100	4(12)	12(36.5)	17(51.5)		29(88)	0	4(12)	
Blast in P.B								
< 50 %	5(16)	6(19.4)	20(64.6)	0.87	26(84)	1(3.)	4(13)	0.7
≥ 50 %	6(16.2)	9(24.3)	22(59.5)		28(75.6)	2(5.4)	7(19)	
Blast in B.M								
< 50 %	2(15.4)	4(30.8)	7(53.8)	0.86	9(69.2)	0	4(30.8)	0.08
≥ 50 %	6(10.5)	20(34.5)	32(55)		52(89.7)	1(1.7)	5(8.6)	
Lineage								
B	17(17.3)	29(29.6)	52(53.1)	0.47	82(83.7)	3(3.1)	13(13.2)	0.67
T	2(15.4)	6(46.2)	5(38.4)		12(92.3)	0	1(7.7)	
CRD (days)	893±657	941±484	1003±490	0.5	958±470	454±415	1173±668	0.07
CR rate	(73.3)	(79.4)	(76.6)	0.8	(77.8)	(50)	(80)	0.4
Risk groups								
SR	3(10)	9(30)	18(60)	0.60	24(80)	0	6(20)	0.43
IR	16(18.6)	26(30)	44(51.4)		70(81.4)	4(4.7)	12(13.9)	
HR	2(7.7)	9(34.6)	15(57.7)		23(88.5)	0	3(11.5)	

All parameters are in number (percentage). Values for age, white blood cell (WBC), platelet, CRD and survival duration are mean ± SD. *PB* peripheral blood; *BM* bone marrow; *CRD* complete remission duration; *CR* complete remission; *SR* standard risk; *IR* Intermediate risk; *HR* high risk

Discussion

Acute leukemia is a cancer of hematopoietic tissue that is characterized by abnormal and uncontrolled proliferation of precursor cells [22]. Based on cellular origin, acute leukemias are classified into myeloid and lymphoid type. AML is more common in adult and ALL in pediatrics. The etiology of leukemia is unknown but several evidences suggest the involvement of environmental and genetic factors in development of the disease [22].

The role of Fas in leukemogenesis has been shown in cancer patients with myeloid and lymphoid- lineage malignancies [23]. This molecule is expressed by many nucleated cells and plays a crucial role in differentiation, regulation and development of myeloid and lymphoid cells [9]. The physiological ligand for Fas, Fas ligand (FasL), is

Table 5 Relationship between extramedullary involvement and hepatomegaly with different genotypes at Fas gene position −670

Data are presented as number (%). *EMI* extramedullary involvement

Prognostic factor		Total patients	Genotype			P value
			GG	AA	AG	
EMI	No	35(34.3)	6(50)	13(41.9)	16(27.1)	0.17
	Yes	67(65.7)	6(50)	18(58.1)	43(72.9)	
Hepatomegaly	No	64(64)	11(91.7)	21(70)	32 (55.2)	0.04
	Yes	36(36)	1(8.3)	9 (30)	26 (44.8)	

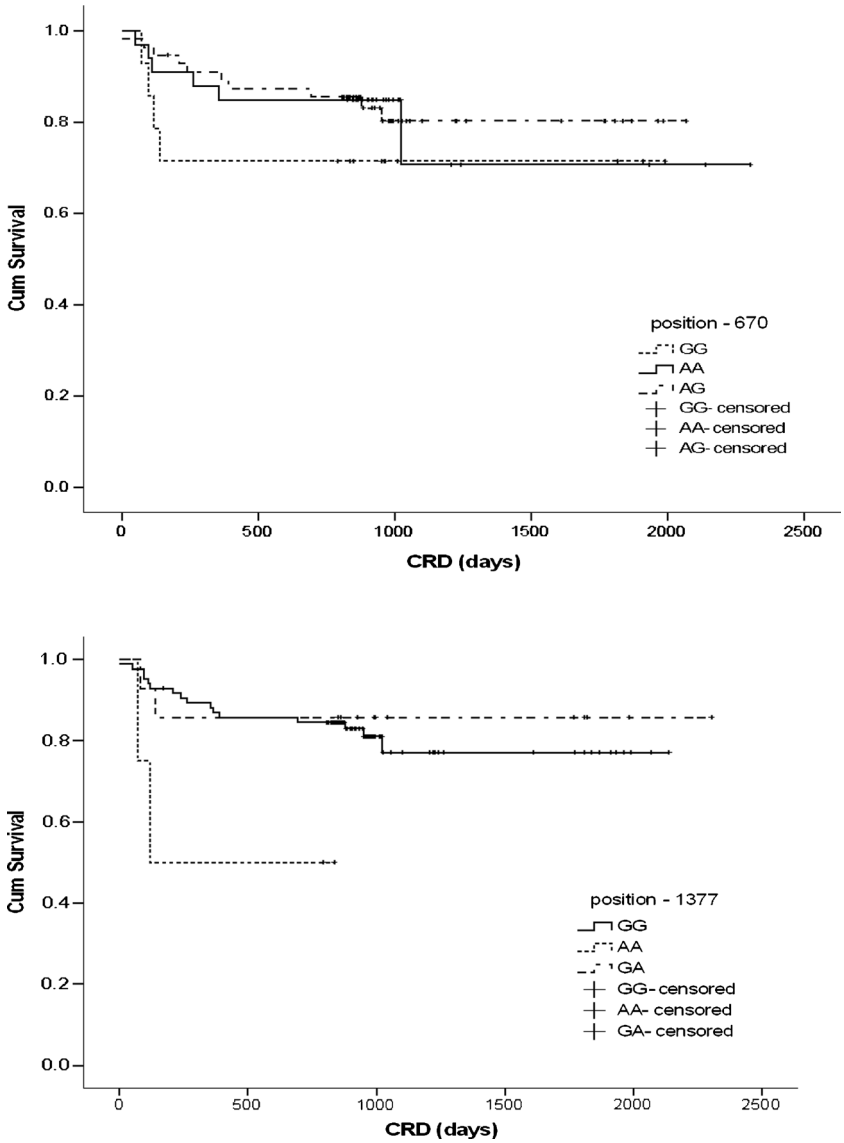

Fig. 2 Kaplan-Meier complete remission duration (CRD) curve for ALL patients in relation to −670 and −1377 genotypes. No significant differences were observed between the groups ($p = 0.07$ for −1377 and $p = 0.5$ for −670 variants calculated by log-rank test)

expressed by activated T cells and non-T cells under a variety of conditions [24]. The interaction of Fas and FasL causes cross-linking of Fas and then this in turn transduces an active signal for cell death [25]. Fas gene promoter polymorphism can be an important genetic risk factor in drug resistance and the chemotherapeutic response of cancer cells [26]. In this regard, several mutations in Fas gene have been reported in multiple myeloma and non-Hodgkin's lymphoma [10, 19]. These mutations have been involved in chemoresistance, leading to escape of tumor cells and contributed to tumorigenesis. Some rare mutations in ATL and T-ALL have also been reported [27]. Beltinger et al. have shown the occurrence of mutations in leukemic blasts of some T-ALL patients (in exon 3 of CD95 causing a 68Pro −>68Leu change associated with decreased CD95-mediated apoptosis) [28]. The same author reported no mutations in the coding and proximal promoter region of Fas gene in 32 primary B-lineage ALL of childhood [29]. In the Fas promoter region, the two positions −670 and −1377, due to their binding capacity to transcription factors and regulating the level of gene expression have been studied in various populations [11]. In the present study, the association between these functional polymorphisms of Fas gene and risk of developing childhood ALL has been investigated. Results of our study indicated no association of Fas genotypes at positions −670 and −1377 bp in the promoter region with increased risk of ALL in children. To the best of our knowledge there are no similar studies on these polymorphisms in ALL patients. However, in limited studies, Fas allele and genotype frequencies in relation to other leukemias have been investigated. Sibley and colleagues have indicated a significantly increased risk of developing AML in adult

patients with heterozygous GA and homozygous AA genotypes at position −1377 bp [9]. In contrast, in a case-control study on Korean population no significant association between Fas polymorphism (−1377G/A) and AML risk has been observed [30]. Study of Fas promoter polymorphism on outcome of children with AML also revealed no significant influence on survival and relapse rates between patients with Fas −1377GG genotype versus −1377 GA/-1377 AA genotypes [31]. In another study, −670 Fas promoter A/G polymorphism was significantly associated to susceptibility, clinical manifestation, and survival in thirty-one patients with ATL, indicating the role of Fas in the disease pathogenesis [20].

The Fas gene promoter polymorphisms were analyzed with respect to established prognostic factors. Prognostic factors can be used as clinical or biologic features to estimate the chance of recovery and treatment options for disease [32–34]. We found a negative correlation between −670 GG genotype and liver involvement, as the frequency of this genotype was lower in patients with hepatomegaly than other patients. From this data one can concluded that this genotype is associated with a better prognosis in ALL patients. However, we believe that due to low number of patients within this genotype the result is not conclusive and additional studies on a higher number of patients is required. There have been inconsistent results regarding Fas expression affected by the −670 position [9]. It can be assumed that this genotype by affecting normal host cells involved in tumor response can enhance the elimination of leukemic cells in peripheral tissues like liver. No significant associations between allelic and genotype frequencies of Fas gene polymorphism at position −1377 and other prognostic factors including age, WBC and platelet numbers, morphological subtypes and cytogenetic was obtained.

We also examined the CRD and CR rate as prognostic markers to find the impact of Fas genotypes on response to therapy. Although patients with −1377 AA genotype had the shortest mean CRD among the various genotypes, possibly due to low number of these patients no significant results was obtained. In a previous study, carrier status for the −1377A variant has been associated with a significantly worse overall survival in acute promyelocytic leukemia patients [35]. The polymorphism at −1377 has been shown to reduce Fas expression level and therefore resistance to Fas-mediated apoptosis [9]. Further studies with higher numbers of the patients are required to clarify the importance of this genotype as a predictive of disease outcome in ALL.

In our study, there were no significant differences in genotype and allele frequencies between patients and controls with regard to gender. The relatively small number of the patients and controls due to the difficulty of obtaining sample from children could be mentioned as a limitation of this study. Also, all clinical and laboratory data were not available for our patient cohort.

In conclusion, Fas polymorphism at positions −670 and −1377 showed no significant impact on susceptibility to ALL. However, association of −670 GG genotype with a lower frequency of the liver involvement in the patients suggests the prognostic value of this genotype in ALL.

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Conflicts of interest The authors declare no conflicts of interest.

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