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Prognostic Importance of Single-Nucleotide Polymorphisms in IL-6, IL-10, TGF-β1, IFN-γ, and TNF-α Genes in Chronic Phase Chronic Myeloid Leukemia

Mustafa Pehlivan,[1] Handan Haydaroglu Sahin,[1] Sacide Pehlivan,[2] Kursat Ozdilli,[2,3] Leylagul Kaynar,[4] Fatma Savran Oguz,[2] Tugce Sever,[1] Mehmet Yilmaz,[1] Bulent Eser,[4] Yeliz Duvarci Ogret,[2] Cem Kis,[1] Vahap Okan,[1] Mustafa Cetin,[4] and Mahmut Carin[2]

The aim of this study was to explore the association between polymorphisms of five cytokine genes and clinical parameters in patients with Philadelphia-positive (Ph+) chronic myeloid leukemia (CML) treated with imatinib. We analyzed five cytokine genes (interleukin [IL]-6, IL-10, gamma interferon [IFN-γ], transforming growth factor beta-1 [TGF-β1], and tumor necrosis factor-alpha [TNF-α]) in 60 cases with Ph+ CML and 74 healthy controls. Cytokine genotyping was performed by the polymerase chain reaction-sequence-specific primer. All data were analyzed using the de Finetti program and SPSS version 14.0 for Windows. No significant differences were detected between the CML group and healthy controls with respect to the distributions and numbers of genotypes and alleles in TNF-α, TGF-β1, IL-10, and IFN-γ. However, the GG genotype associated with high expression in IL-6 was found to be significantly more frequent in CML as compared to controls ($p = 0.010$). The median follow-up time was 49.3 months (range 6.1–168.4) and the median duration of imatinib treatment was 39.5 months (range 5.2–103.4) for these patients. On multivariateanalysis, only IL-10 GCC/GCC highly produced haplotypes were significantly associated with a shorter event-free survival. The relationship between cytokine genotypes/haplotypes and clinical parameters in CML has not been investigated before. Our results suggest that IL-10 may be a useful marker for CML prognosis and theGG genotype of the IL-6 gene may be associated with susceptibility.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of clonal origin with an annual incidence of about 1 in 50,000 (Chen and Li, 2013). CML patients usually present in the chronic phase of the disease, during which there is a gradual expansion of mature myeloid cells in the bone marrow and peripheral blood. Without treatment, patients inevitably progress through an accelerated phase of disease (4–6 years on average after diagnosis) to a terminal acute phase known as blast crisis, which is characterized by a massive increase in undifferentiated blasts that can be either myeloid or lymphoid in nature (Mayani *et al.*, 2009). In their study, Nowell and Hungefort (1960) described the origin of CML as a common chromosomal abnormality that they found in CML patients and suggested a ‘‘causal relationship between the chromosomal abnormality observed and chronic granulocytic leukemia.’’ This unique chromosomal abnormality, known as the Philadelphia chromosome (Ph), was later shown to be a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9:22)(q34;q11)] and leads to the fusion of the breakpoint cluster region (BCR) and human ABL1 genes (Rowley, 1973). The resulting BCR-ABL fusion gene codes for BCR-ABL transcripts and fusion proteins with unregulated tyrosine kinase activity (Hochhaus, 2008). Since 1998, patients with Ph-positive (Ph+) CML have been treated successfully with the abl-tyrosine kinase inhibitor, imatinib. More than 95% of the patients have achieved a complete hematologic response, and more than 80% have achieved a complete cytogenetic response (CCR) (Aliano *et al.*, 2013). However, a proportion of patients demonstrate resistance or suboptimal response to imatinib therapy; in many cases, the mechanism is unknown (Aliano *et al.*, 2013; Chen and Li, 2013).

[1]Department of Hematology, Faculty of Medicine, Gaziantep University, Gaziantep, Turkey.
[2]Department of Medical Biology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey.
[3]Medipol University Hospital, Istanbul, Turkey.
[4]Department of Hematology, Faculty of Medicine, Erciyes University, Kayseri, Turkey.

The initiating event in the pathogenesis of CML is the well-known chromosomal translocation (9;22) that drives the aberrant differentiation of hematopoietic stem cell prefentially toward the myeloid lineage. Although necessary, BCR-ABL expression alone is not sufficient for the progression from chronic phase to accelerated phase or blast crisis CML. BCR-ABL expression persists in the bone marrow of patients who have achieved and maintained CCR for up to 10 years (Chomel *et al.*, 2000). It has also been detected in leukocytes of healthy normal persons who never go on to develop CML (Bose *et al.*, 1998). These findings would suggest that additional different gene mutations are needed for progression to leukemia to occur (Rise and Jamieson, 2010). It has been hypothesized that genetic factors other than histocompatibility disparity could play a role in the predisposition or prognosis for CML. In this regard, T helper types 1 and 2 (Th1 and Th2), cytokines, and the polymorphisms in their genes seem to be important (Humlová *et al.*, 2006). Cytokines released from activated lymphocytes, monocytes, and macrophages modify the intensity of the immune inflammatory response. These molecules function as chemical mediators among cells and are recognized by specific receptors on the target cells. In addition to supporting the immune response locally and systemically, cytokines also regulate hematopoiesis. Differences in cytokine production are related to sequence variants in their genes. Previous studies have shown that polymorphisms in regulatory regions of cytokine genes could affect transcription, resulting in variations of cytokine protein production. Recently, the association between cytokine genes and leukemias has been examined intensively (Bidwell *et al.*, 2001; De Oliveira, 2007).

In this study, it was aimed (1) to investigate single-nucleotide polymorphisms in five different cytokine genes in 60 chronic phase Ph+ CML patients and 74 healthy controls followed between 2000 and 2008 and (2) to compare the correlations of these results with clinical data and determine their effects on total and progression-free life durations.

Table 1. Clinical Features of Chronic Myeloid Leukemia in Chronic Phase Patients

	n *(%)*
Number of patients	60
Age at diagnosis	41 (20–74)[a]
Age ≥60 years	6 (10)
Male/female	23/37 (38.3/61.7)
Splenomegaly	42 (70)
Hemoglobin 12< g/dL	42 (70)
Leukocytes $>50\times10^9$/L	38 (63.3)
Platelets $>450\times10^9$/L	28 (40)
Sokal risk score at diagnosis	
Low	10 (16.7)
Intermediate	28 (46.7)
High	22 (36.6)
Initial treatment	
Imatinib 400 mg/day	50 (83.3)
Interferon-α→imatinib 400 mg/day	10 (16.7)
Response ELN criteria (18 months)	
Optimal	40 (66.6)
Suboptimal	13 (21.7)
Failure	7 (11.7)
Mortality	2 (3.3)
Event[b]	12 (20)
Chromosomal abnormalities in addition to the Philadelphia chromosome	4 (6.7), trisomy 8 [2](3.4), monosomy 7 [1](1.7), trisomy 21[1](1.7)
Time after diagnosis, months	49.3 (6.1–168.4)[a]
Duration of imatinib, months	39.5 (5.2–103.4)[a]

[a]Median.
[b]Death (2), progression to accelerated phase or blastic phase (2), loss of an MCyR (8).
ELN, European Leukemia Net.

Materials and Methods

The study included 74 controls (ethnically matched healthy unrelated individuals), and 60 patients diagnosed with chronic phase Ph+ CML were diagnosed at the Department of Hematology, Medical School Hospital, Gaziantep and Erciyes Universities, Turkey. The study was approved by the Local Ethics Committee. Written informed consent was received from all participants. Of 60 patients with CML, 23 were male and 37 were female. The age of these patients ranged from 20 to 74 years. Ten patients (16.7%) were designated as the low-risk group, 28 patients (46.7%) as the intermediate-risk group, and 22 patients (36.6%) as the high-risk group, according to the Sokal risk score assigned at diagnosis (Table 1) (Sokal *et al.*, 1984). All 60 patients with Ph+ CML in chronic phase (within 1 year of diagnosis) were treated with 400 mg oral imatinib daily. Ten of those patients were also treated with a gamma interferon (IFN-γ) protocol (O'Brien *et al.*, 2003; Aliano *et al.*, 2013). Genomic DNA was extracted from mononuclear cells obtained from EDTA-treated peripheral venous blood using the salting-out method (Miller *et al.*, 1988). Cytokine genotyping was performed by the polymerase chain reaction-sequence-specific primer method, using the Cytokine Genotyping Tray kit according to the manufacturer's instructions (One Lambda). Single-nucleotide polymorphisms for five cytokines (interleukin [IL]-6, IL-10, IFN-γ, transforming growth factor beta-1 [TGF-β1], and tumor necrosis factor-alpha [TNF-α]) were analyzed (Karaoglan *et al.*, 2009). All data were analyzed using SPSS version 14.0 for Windows (SPSS, Inc.). Categorical data were analyzed using Pearson's chi-square analysis. Odds ratio (OR) and 95% confidence interval (95% CI) were also calculated. OR (95% CI) was adjusted by age and sex. The data were analyzed for appropriateness between the observed and expected genotypes as well as for Hardy–Weinberg Equilibrium (HWE) as described elsewhere. All analyses were two tailed, and differences were interpreted as statistically significant when $p<0.05$.

Results

Clinical features of the chronic phase CML patients are given in Table 1. The patients were between 20 and 74 years old and the median age was 41. The median follow-up time was 49.3 months (range 6.1–168.4), and the median duration of imatinib treatment was 39.5 months (range 5.2–103.4) for these patients. Table 2 shows genotype distributions for patients and controls. No significant difference was detected between the CML patients and controls for TNF-α (−308), IL-10 (−592, −819, −1082), INF-γ (+874), and TGF-β1

Table 2. Comparison of Frequencies of TNF-α, TGF-β, IL-10, IL-6, and IFN-γ Gene Polymorphisms Between Patients with Chronic Myeloid Leukemia and Healthy Controls

	Genotype	CML		Healthy control		OR	95% CI	p
		n[a]	(%)	n[b]	(%)			
TNF-α (−308)	GG	56	(93.3)	66	(89.2)	0.621[c]	0.158–2.445[c]	0.496[c]
	AG	3	(5)	8	(10.8)	0.540[c]	0.126–2.319[c]	0.407[c]
	AA	1	(1.7)	–	(0)	0.444[d]	0.367–0.537[d]	0.448[d]
TGF-β (codons 10)	CC	12	(20)	11	(14.9)	1.387[c]	0.445–4.228[c]	0.565[c]
	TC	32	(53.3)	40	(54.1)	1.161[c]	0.485–2.776[c]	0.738[c]
	TT	16	(26.7)	23	(31)	1215[c]	0.528–2.797[c]	0.647[c]
TGF-β (codons 25)	GG	52	(86.6)	59	(79.7)	0.597[c]	0.220–1.621[c]	0.312[c]
	GC	7	(11.7)	15	(20.3)	0.507[c]	0.180–1.431[c]	0.199[c]
	CC	1	(1.7)	–	(0)	0.444[d]	0.367–0.537[d]	0.448[d]
IL-10 (−1082)	AA	31	(51.7)	28	(37.8)	1.152[c]	0.350–2.794[c]	0.816[c]
	AG	22	(36.7)	36	(48.6)	0.882[c]	0.264–2.947[c]	0.838[c]
	GG	7	(11.6)	10	(13.6)	0.895[c]	0.247–3.237[c]	0.865[c]
IL-10 (−819)	CC	25	(41.7)	37	(50)	1.305[c]	0.237–7.187[c]	0.759[c]
	CT	32	(53.3)	33	(44.6)	1.981[c]	0.359–10.948[c]	0.433[c]
	TT	3	(5)	4	(5.4)	2.851[c]	0.434–18.743[c]	0.276[c]
IL-10 (−592)	CC	25	(41.7)	37	(50)	1.305[c]	0.237–7.187[c]	0.759[c]
	CT	32	(53.3)	33	(44.6)	1.981[c]	0.359–10.948[c]	0.433[c]
	TT	3	(5)	4	(5.4)	2.851[c]	0.434–18.743[c]	0.276[c]
IL-6 (−174)	GG	47	(88.3)	37	(50)	0.347[c]	0.155–0.774[c]	0.010[c]
	GC	7	(11.7)	32	(43.2)	0.219[c]	0.083–0.579[c]	0.002[c]
	CC	6	(10)	5	(6.8)	0.687[c]	0.177–2.657[c]	0.586[c]
IFN-γ (+874)	TT	9	(15)	9	(12.2)	0.700[c]	0.236–2.079[c]	0.520[c]
	TA	31	(51.7)	32	(43.2)	0.879[c]	0.279–2.767[c]	0.826[c]
	AA	20	(33.3)	33	(44.6)	0.529[c]	0.163–1.721[c]	0.290[c]

[a] $n=60$.
[b] $n=74$.
[c] OR (95% CI) was adjusted by age and sex.
[d] Fisher's exact test.
95% CI, 95% confidence interval; IFN-γ, gamma interferon; IL, interleukin; OR, odds ratio; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-alpha; CML, chronic myeloid leukemia.

(codons 10 and 25) polymorphisms ($p>0.05$). However, the genotype for IL-6 (−174) was significantly different between the patients and the controls. The 6-year probability of overall survival (OS) was 96% and event-free survival (EFS) 73% in all patients (Tables 1 and 3). In the univariate analyses, there was no significant prognostic factor found to influence OS (Table 3). The five factors that were predicted for better EFS from the univariate analysis were younger age (<60) ($p=0.023$), absence of splenomegaly ($p=0.014$), lower Sokal risk score at diagnosis ($p=0.018$), TGF-β1 haplotype of TCGC, TTGC, CCGC, CCCC, CCGG, or TCCC ($p=0.019$), and IL-10 haplotype of GCC/ACC, GCC/ATA, ACC/ACC, ACC/ATA, and ATA/ATA ($p=0.002$) (Fig. 1). However, in the multivariate analysis, only the IL-10 (−1082, 819, 592) GCC/GCC haplotype was significantly associated with lower EFS (Fig. 2). The Cox proportional hazard ratio was 0.140 ($p=0.019$, [95% CI: 0.027–0.726]).

Discussion

In this work, we investigated certain cytokine gene polymorphisms in CML patients. Previous studies have indicated the role of immunologic responses in the mechanisms of prognosis, sepsis, and mortality during CML therapy (Bidwell *et al.*, 2001; Humlová *et al.*, 2006; Hochhaus, 2008). It is thought that increased cytokine levels and complement activation may be responsible for CML (Amirzargar *et al.*, 2005). In addition to its important role in immune response and inflammatory processes, the cytokine IL-6 is crucially involved in carcinogenesis (www.ncbi.nlm.nih.gov/omim?term=IL6). IL-6 is associated with poor prognosis in various malignancies (Deans *et al.*, 2007; DeMichele *et al.*, 2009). Nevertheless, it was reported that this cytokine was not associated with multiple myeloma or primary cutaneous melanoma (Martinez-Escribano *et al.*, 2003; Duch *et al.*, 2007). No data are available on a possible association with CML. When the CML and control groups were compared in this study, the IL-6 (−174) polymorphism exhibited a significant difference both in terms of allele frequency and genotype frequency; there was a deviation from HWE in the CML group as well (Table 2). The most frequent genotype in our CML patients was IL-6 GG at position −174 (88.3% CML vs. 50% control, $p=0.010$). In contrast, the frequencies of the genotype IL-6 GC at position −174 (11.7% vs. 43.2%, $p=0.002$) were very low in the CML patients. This substitution could result in high levels of IL-6 secretion. The high expression of IL-6 in the results is in accordance with the literature (Martinez-Escribano *et al.*, 2003; Lehrnbecher *et al.*, 2005; Snoussi *et al.*, 2005; Deans *et al.*, 2007; Ambruzova *et al.*, 2009; Bhattacharyya *et al.*, 2009).

TGF-β1, a multifactorial cytokine, is the strongest known growth inhibitor of normal and transformed cells (Deans *et al.*, 2007; Noori-Daloii *et al.*, 2007). Increased TGF-β1 expression and EGFR amplification accompany the emergence

Table 3. Univariate Analysis (Logrank Test) of Prognostic Factors in 60 Patients with Chronic Phase CML

	n	*6 years OS %*	*Log rank* p-*value*	*6 years EFS % (median months)*	*Log rank* p-*Value*
All patients	60	96		73	
Gender					
Female	37	97		71	
Male	23	96	0.705	75	0.708
Age					
<60	54	96		77	
≥ 60	6	100	0.643	40 (25.8)	0.023
Sokal risk score at diagnosis					
Low	10	100		100	
Intermediate	28	100	0.148	85	0.018
High	22	90		38 (53.8)	
Low–intermediate–high	38/22	100/90	0.050	87/38 (53.8)	0.007
Splenomegaly					
Yes	42	95		61	
No	18	100	0.353	100	0.014
Hemoglobin (g/dL)					
<12	42	95		60	
≥ 12	18	100	0.353	87	0.144
Leukocytes ($\times 10^9$/L)					
<50	22	100		89	
≥ 50	38	94	0.276	61	0.087
Platelets ($\times 10^9$/L)					
<450	32	97		82	
≥ 450	28	96	0.930	65	0.392
Initial treatment					
Imatinib	50	96		73	
Interferon-α → imatinib	10	100	0.525	77	0.685
TNF-α (−308)					
GG[a]	56	96		77	
GA/AA[b]	4	100	0.717	50 (53.8)	0.943
TGF-β (codons 10, 25)					
TCGC, TTGC, CCGC, CCCC, CCGG, TCCC[a,c]	26	100		100	
TTGG, TCGG[b]	44	95	0.372	63	0.019
IL-10 (−1082, 819, 592)					
GCC/ACC, GCC/ATA, ACC/ACC, ACC/ATA, ATA/ATA[a,c]	52	98		77	
GCC/GCC[b]	8	88	0.085	42 (25.8)	0.002
IL-6 (−174)					
CC[a]	6	100		100	
GG/GC[b]	54	96	0.643	70	0.198
IFN-γ (+874)					
AA/TA[a,c]	51	96		82	
TT[b]	9	100	0.552	42 (53.8)	0.083

Sokal, patient's age, spleen size, percentage of blood blasts and platelets.
[a]Low production.
[b]High production.
[c]Intermediate production.
EFS, event-free survival; OS, overall survival.

of highly aggressive human carcinomas. The literature contains data supporting the fact that the polymorphisms of this gene could be associated with susceptibility to the disease, but contrasting data in the literature suggest that such polymorphisms could be protective (Noori-Daloii *et al.*, 2007; Castillego *et al.*, 2009; Hawinkels *et al.*, 2009; Qian *et al.*, 2009). No associations were detected between TGF-β1 genotype/allele frequencies and CML in our study. However, the low to intermediate production of TGF-β1 was shown to improve EFS in univariate analysis, and no associations were found in multivariate analysis. No similar analyses were performed up to date.

IL-10 is a multifunctional cytokine with both immunosuppressive and antiangiogenic functions and could have both tumor-promoting and tumor-inhibiting properties. A large number of polymorphisms (primarily single-nucleotide polymorphisms) have been identified in the IL-10 gene promoter (Martinez-Escribano *et al.*, 2003; Wilkening *et al.*,

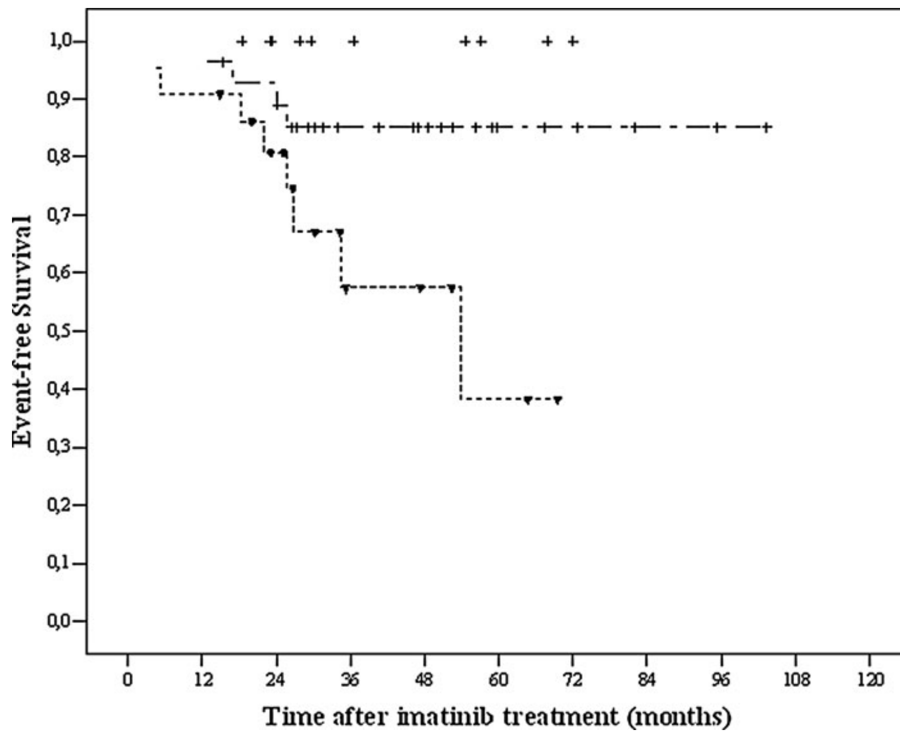

FIG. 1. Kaplan–Meier plots on event-free survival (EFS) time according to the type of Sokal risk score at diagnosis. Low risk 100% ($n=10$). Intermediate risk 85% ($n=28$). High-risk median 53.8 months, 38% ($n=22$). Log rank $p=0.018$.

2008). Convincing evidence that all of these polymorphisms are associated with differential expression of IL-10 *in vitro*, and in some cases *in vivo*, was obtained, and a number of studies investigated associations between IL-10 polymorphisms and cancer susceptibility and prognosis (Martinez-Escribano *et al.*, 2003; Cacev *et al.*, 2008; Wilkening *et al.*, 2008). However, because of the marginal success of the initial clinical trials using recombinant IL-10, some of the interest in this cytokine as an anti-inflammatory therapeutic agent has diminished (Hawinkels *et al.*, 2009). IL-10 was reported to be a poor prognostic factor in colorectal, gastro-esaphageal, and sporadic colon cancers and advanced melanoma, while no associations were reported between IL-10 and nasopharyngeal or breast carcinoma (Martinez-Escribano *et al.*, 2003; Pratesi *et al.*, 2006; Vuoristo MS, 2007; Bogunia-Kubik *et al.*, 2008). In our study, no associations could be detected between IL-10 and CML in terms of allele/genotype frequency. Low to intermediate production of IL-10 was

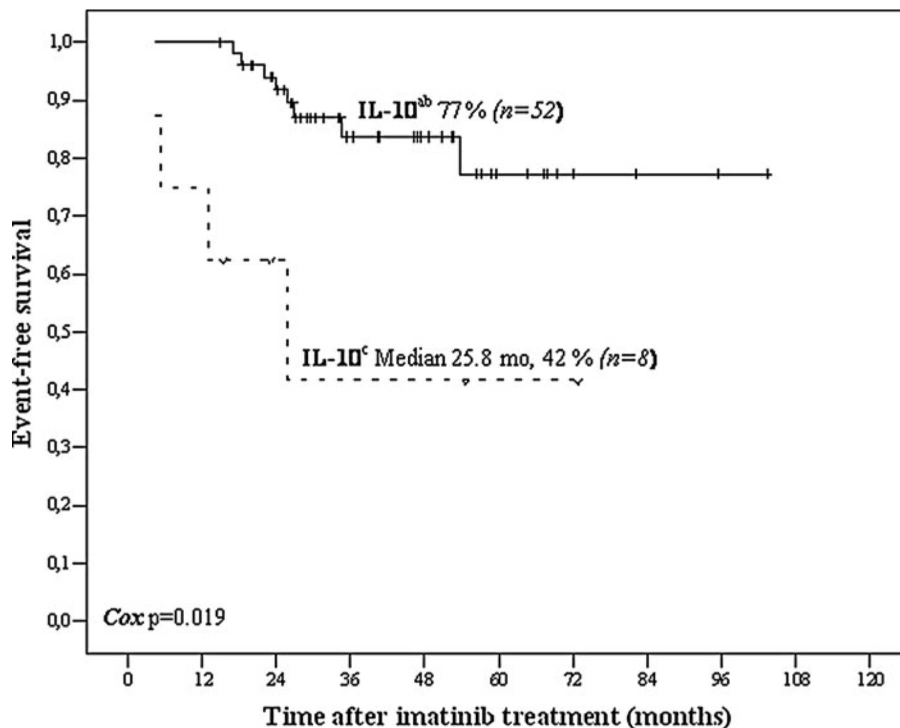

FIG. 2. Kaplan–Meier plots on EFS time according to the type of interleukin (IL)-10 (−1082, 819, 592) haplotypes. [ab], Low and intermediate production haplotypes (GCC/ACC, GCC/ATA, ACC/ACC, ACC/ATA, ATA/ATA), [c], high production haplotypes (GCC/GCC).

demonstrated to be associated with better EFS in the univariate analysis, and high production of IL-10 was found to be associated with lower EFS in the multivariate analysis (Fig. 2). Previous studies indicate that IL-10 could be a preferred survival marker, and our results support this fact (Hempel *et al.*, 1997; Martinez-Escribano *et al.*, 2003; Amirzargar *et al.*, 2005).

Genetic polymorphisms in the promoter region of the TNF-α gene are involved in the regulation of its expression levels and have been associated with various inflammatory and malignant conditions (Jevtovic-Stoimenov *et al.*, 2008; Castillego *et al.*, 2009). Previous studies demonstrated that TNF-α −308 polymorphism is not associated with chronic lymphocytic leukemia (Au *et al.*, 2006). Also, there is no association between TNF-α and myeloma/myelodisplastic syndrome (Gyulai *et al.*, 2005; Au *et al.*, 2006). Although TNF-α polymorphism association in colorectal and NSCLC has been reported, there are no studies on the association between TNF-α polymorphisms and CML in the literature (Colakogullari *et al.*, 2008; Wu *et al.*, 2008). This is the first study in this field, and it was shown that TNF-α polymorphisms do not play a role in the prognosis of CML.

IFN-γ is a proinflammatory cytokine playing a pivotal role in both innate and adaptive immune responses (Halma *et al.*, 2004). A significant association has been reported between IFN-γ polymorphisms and cervical and pancreatic cancers (Halma *et al.*, 2004; Gangwar *et al.*, 2009), whereas no associations have been detected for breast, lung, and cervical cancers in particular populations (Halma *et al.*, 2004; Gonullu *et al.*, 2007; Colakogullari *et al.*, 2008). Also, no significant associations were detected between allele/genotype frequencies and clinical parameters in our CML patient group.

In conclusion, the relationship between cytokine polymorphisms and clinical parameters in Ph+ CML was investigated in this study for the first time. Our results suggest that IL-10 could be a useful marker for CML prognosis and IL-6 polymorphisms could be associated with susceptibility. These results must be replicated in larger populations, which are needed to elucidate the role of IL-6 and IL-10 in CML.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Sacide Pehlivan, MSc, PhD
Department of Medical Biology
Istanbul Faculty of Medicine
Istanbul University
Capa, Fatih
Istanbul 34080
Turkey

E-mail: sacide.pehlivan@istanbul.edu.tr; psacide@hotmail.com