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Endothelial Nitric Oxide Synthase Gene Variants and Susceptibility to Chronic Myeloid Leukemia (Ph+)

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ABSTRACT The aim is to study the relationship of Philadelphia positive (Ph+) - Chronic Myeloid Leukemia (CML) with endothelial nitric oxide (NOS3) gene variants (intron 4 VNTR and 894G> T). Two NOS3 gene variants were evaluated in 59 CML patients and 100 healthy controls. Variants for the NOS3 gene were detected by PCR and/or PCR-RFLP. When compared with the healthy control group (p: 0.000 and 0.011, respectively), the researchers found that NOS3 (894G>T) TT genotype and T allele were higher in the patients with CML. The researchers found that NOS3 (intron 4 VNTR) BB genotype and B allele were higher in the patients with CML in comparison with the healthy control group (p: 0.040 and 0.046, respectively). Regarding haplotype frequency, haplotypes 33, 32, 31 and 23 (BBTT, BBGT, BBGG and ABTT) were found to be existing in CML only. The researchers demonstrated that these two variants could play a role in the ethiopathogenesis of CML.

INTRODUCTION

The accumulated "mutations" in genes which have a role in control cellular growing and specialization leads to disease progression in myeloid malignancies. In myeloid diseases, genetic alterations have been found. It is suggested through the evidences that the production of endogenous sources of DNA damage increases and/or decreases; it produces reactive oxygen species resulting in the genetic changes in myeloid malignancies (ROS) (Sallmyr et al. 2008; Kotagama et al. 2015). Nitric oxide (NO) is an endogenous molecule with a physiological and pathophysiological role in cancer biology. Low NO concentrations (picocene-nanomolar range) have tumor-promoting, high NO concentrations (micromolar range) pro-apoptotic functions. It causes tumor suppression due to its pro-apoptotic function and is involved in immunosuppressive therapy (Seabra and Duran 2018).

Nitric oxide (NO), synthesized from L-arginine, is a free radical and nitric oxide synthase catalyzes it. NO has three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS or NOS3) and inductive NOS (iNOS). Large NO concentrations are produced by iNOS for a longer period of time (Kashif and Duvalsaint 2017). The eNOS is encoded by a gene located on chromosome 7q35-36, consisting of 26 exons and covering a genomic region of 21 kb. (NO2). There are three common polymorphisms of this gene described as the T-786C in the promotor region, the missense E298D (894 G>T) in exon 7, and the 27-bp repetition in intron 4 (Kim et al. 2012; Chaaben et al. 2015). NO has been comprised in the pathogenesis of several diseases like Chronic Myeloid Leukemia (CML) through the excessive production of ROS as well as through its genetic polymorphism (He et al. 2014; Yanar et al. 2016). Owing to the inclusion of endogenous NO and eNOS in human cancers such as acute myeloid leukemia (AML), CLL, breast cancer, nasopharyngeal carcinoma, and larynx cancer (Hao et al. 2010; He et al. 2014; Chaaben et al. 2015; Yanar et al. 2016; Pehlivan et al. 2017), it is supposable that eNOS gene variants may be important for CML.

European CML reports consistently median between 0.1-1.0/100,000 per year, average age at diagnosis is 57-60 and has between 1.2-1.7 years male/female ratio. There has been a consistency of incidence of CML over time. Although there is not exact knowledge about the prevalence of CML, between 10-12/100,000 is the estimated number. In recent population-based studies, survival has been reduced in CML patients over 70 years, but overall survival has been observed in large clinical trials (Höglund et al. 2015).

CML patients are mostly in chronic phase which has a graded condition of the mature cells of myeloid in the peripheral blood and bone marrow. Terminal acute phase is seen 4-6 years after diagnosis in patients if not treated and it is qualified as a large rise in uniform blasts, which might be lymphoid or myeloid (Kotagama et al. 2015).

Nowell and Hungerford (1960) identified a standard chromosomal abnormality in CML patients and important information about CML was obtained from this work. CML is characterized by the existence of chimeric BCR-ABL oncogen (Chereda and Melo 2015; Zhou and Xu 2015; Bennour et al. 2016). The molecular mechanisms underlying CML are not completely understood. It is known that increased intracellular reactive oxygen species and spontaneous DNA damage are associated with the activity of BCR-ABL (Annamaneni et al. 2013).

At the center of cell biology of CML is BCR-ABL1 (Chereda and Melo 2015). For this reason, the diagnosis and monitoring of the disease involves the detection of BCR-ABL1 (Chereda and Melo 2015; Kotagama et al. 2015).

Objectives

The aim of this study is to research the NOS3 gene variants (894G>T and intron 4 VNTR) in CML (Ph+) patients and healthy controls.

MATERIAL AND METHODS

Subjects

The study included 100 healthy control subjects matched for age and sex and 59 patients who have Ph+CML in chronic phase at Erciyes and Gaziantep Universities, Medical School Hospital, the Department of Hematology. Local Ethics Committee has approved the study. The total of 59 patients were determined as 23 males and 36 females aging from 20 to 74 years. According to Sokal risk score at diagnosis (Table 1), 22 patients (36.6%) were placed in the high-risk group, 27 patients

Table 1: Clinical features of CML in chronic phase patients

			n (%)	
Number of Patients		59		
Age at Diagnosis		41	(20-74)*	
Age > 60 years		6	(10.2)	
Male/Female		23/36	(39/61)	
Splenomegaly		42	(71.2)	
Hemoglobin 12<g/dL		42	(71.2)	
Leukocytes > 50 x 10^9/L		38	(64.4)	
Platelets > 450 x 10^9/L		28	(47.6)	
Sokal Risk Score at Diagnosis	Low risk	10	(16.9)	
	Intermediate risk	27	(45.8)	
	High risk	22	(37.3)	
Initial Treatment	Imatinib 400 mg/d	50	(84.7)	
	Interferon-á'!imatinib 400 mg/d	9	(15.3)	
Response ELN Criteria (18mo)	Optimal	40	(67.8)	
	Suboptimal	13	(22)	
	Failure	6	(10.2)	
Total Mortality		2	(3.4)	
Event&		12	(20.3)	
Chromosomal Abnormalities in Addition to the Philadelphia Chromosome		4	(6.7)	Trisomy 8 [2], Monosomy 7 Trisomy 21
*Time after Diagnosis, mo**		49.3	(6.1-168.4)*	
*Duration of Imatinib, mo**		39.5	(5.2-103.4)*	

ELN: European Leukemia Net, *median, mo: months, death (2), progression to AP or blastic phase (2), loss of a McyR (8)

(45.8%) in the intermediate-risk group and ten patients (16.7%) in the low-risk group. All the patients with Ph+CML got 400 mg of oral imatinib treatment per day within 1 year of the diagnosis during chronic phase.

DNA Extraction

Miller et al. (1988) suggested that genomic DNA was extracted from the mononuclear cells obtained from the EDTA-treated peripheral venous blood using the salting out method.

NOS3 894 G>T Genotyping

206-bp fragment was amplified by Polymerase chain reaction (PCR) (Erciyas et al. 2010). Fragment was digested with MboI restriction endonuclease (Invitrogen CA, USA) at 37°C overnight. Digestion was resolved on the three percent agarose gel and visualized using ultraviolet light. There was a consistent restriction site for 206-bp PCR products leading to a 119-bp fragment and an 87-bp fragment (Fig. 1). For the internal quality control, twenty percent of the samples were duplicated in order to prevent sample or reading errors.

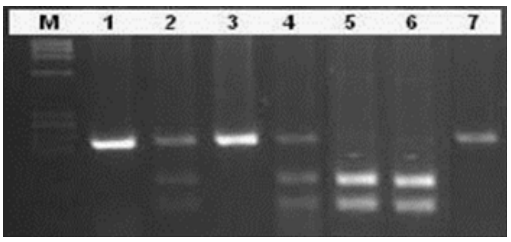

Fig. 1. Agarose gel electrophoresis of NOS3 (894 G>T) DNA fragments stained with ethidium bromide (M: DNA size standard, ND: Non-digest PCR product. The results regarding the patients with CML; 1, 2, 7: GG; 2-3: GT; 5-6: TT)

NOS3 Intron 4 VNTR Genotyping

The researchers designed primers to amplify a 393-bp and/or a 420-bp segment of the NOS3 intron 4 VNTR region which also includes the microsatellite repeat sequence (Erciyas et al. 2010). Then, the researchers separated the products on four percent NuSieve GTG agarose (Fig. 2). For each sample, they repeated the experimental step twice.

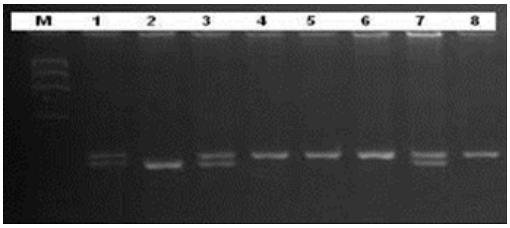

Fig. 2. Agarose gel electrophoresis of NOS3 (VNTR) fragments stained with ethidium bromide (M: DNA size standard. The results regarding the patients with CML; 4-6, 8: AA; 1, 3, 7: AB; 2: BB)

Statistical Analysis

SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL; USA) has been used for the analysis of all data. Pearson's chi-square test and de-Finetti program has been used for the analysis of categorical data. Calculations of the odds ratio (OR) and the ninety-five percent confidence interval (CI) were made. OR (95% CI) was determined through sex and age. The convenience of both between the observed and expected genotypes and Hardy-Weinberg Equilibrium (HWE) were attained through data analysis. Two-tailed analysis were made and when p is less than 0.05 ($p<0.05$.), differences were statistically accepted as significant.

RESULTS

In the study, intron 4 VNTR4 A/B and the G894T variants of eNOS gene were genotyped in adult healthy controls (n=100) and CML patients (n=59) -a total of 159 subjects. Table 1 demonstrates the clinical characteristics of study group.

The distribution of genotypes and HWE were obtained in the CML patients and the control group (Table 2). BB, AB and AA genotype profiles' prevalence for eNOS intron 4 VNTR variant were identified as three percent, twenty-five percent and seventy-two percent respectively in control group and 11.9 percent, 25.4 percent and 62.7 percent respectively in patients with CML. NOS3 (intron 4 VNTR) BB genotype and B allele was found as higher in the patients with CML. In the control group, 0 individuals (0%) were TT genotype, 35 individuals (35%) were GT genotype, and 65 healthy individuals (65%) were GG genotype; in the CML group, 8 patients (13.6%) were TT genotype, 19 patients

Table 2: A comparison of the frequencies of NOS3 (894G>T) and NOS3 (VNTR) gene polymorphisms between the patients with CML and the healthy controls

	Genotype	*CML*	*Control*	*HWE p*	*OR*	*95% CI*	*p*
		n[a] (%)	*n[b] (%)*				
NOS (VNTR)	AA	37 (62.7)	72 (72.0)	0.079[a]	0.219[*]	0.053-0.909[*]	0.037[*]
	AB	15 (25.4)	25 (25)	0.033[b]	0.249[*]	0.055-1.125[*]	0.071[*]
	BB	7 (11.9)	3 (3.0)		0.230[&]	0.057-0.926[&]	0.040[&]
	A	89 (74.4)	169 (84.5)				
	B	29 (24.6)	31 (15.5)		0.563[&]	0.319-0.993[&]	0.046[&]
NOS (894 G>T)	GG	32 (54.2)	65 (65)	0.015[a]	0.638[&]	0.331-1.231[&]	0.184[&]
	GT	19 (32.2)	35 (35)	0.648[b]	1.134[&]	0.572-2.245[&]	0.863[&]
	T T	8 (13.6)	0 (-)		0.338[&]	0.270-0.422[&]	0.000[&]
	G	83 (70.3)	165 (82.5)				
	T	35 (29.7)	35 (17.5)		0.503[&]	0.294-0.861[&]	0.011[&]

[a]n=59, [b]n=100, [*]OR (95% CI) was adjusted by age and sex, [&]Fisher's Exact Test, HWE: Hardy-Weinberg Equilibrium

(32.2%) were GT genotype, and 32 patients (54.2%) were GG genotype for eNOS gene's G894T variant.

Genotypes, haplotypes and allelic frequencies for these genes were compared in the patient and control groups. The researchers found that NOS3 (894G>T) TT genotype and T allele were higher in the patients with CML as compared with the healthy control group (p: 0.000 and 0.011, respectively).

Regarding haplotype frequency, haplotypes have been found as 33, 32, 31 and 23 (BBTT, BBGT, BBGG and ABTT) were present in CML only (Table 3).

DISCUSSION

Despite the rare malignancy, CML has advanced into a model system which includes the study of different aspects of immunology and cancer biology (Vonka and Petráćková 2015).

The importance of eNOS intron 4 A/B variant has been examined in a variety of studies (Celik et al. 2008; Erciyas et al. 2010; Sivri et al. 2014; Nasr et al. 2016). The association of ENOS variants with human cancers has been investigated in previous studies (Yeh et al. 2009; Hao et al. 2010; Ramírez-Patiño et al. 2013; Safarinejad et al. 2013; He et al. 2014; Wu et al. 2014; Haque et al. 2015; Polat et al. 2015; Pehlivan et al. 2017), but there is no study yet on the relationship with CML. The significance of this study is that eNOS variants of CML risk have been evaluated for the first time.

In this study, when the clinical parameters are observed, there is no significant correlation with the clinical parameters (data not shown). The NOS3 894 G>T polymorphism was found as a predicting factor for OS (P = 0.014; hazard ratio = 1.856) by He et al. (2014). However, relapse-free survival, the NOS3 894 G>T polymorphism and relapse in patients with AML (He et al. 2014) were not observed to have a significant association.

Table 3: A comparison of the frequencies of NOS3 haplotypes between the patients with CML and the healthy controls

Haplotypes	*CML*	*Control*	*OR*	*95% CI*	*p[&]*
	n[a] (%)	n[b] (%)			
11	22 (37.3)	52 (52)	1.897	0.982-3.661	0.071
12	11 (18.6)	14 (14)	0.710	0.299-1.687	0.501
13	4 (6.8)	2 (2)	0.281	0.050-1.582	0.196
21	6 (10.2)	20 (20)	2.208	0.832-5.861	0.124
22	6 (10.2)	12 (12)	1.205	0.427-3.400	0.801
23	3 (5.1)	- (0)	0.359	0.291-0.443	0.049
31	4 (6.8)	- (0)	0.355	0.287-0.439	0.018
32	2 (3.4)	- (0)	0.363	0.295-0.447	0.136
33	1 (1.7)	- (0)	0.367	0.299-0.450	0.371

[a]n=59, [b]n=100, [*]OR (95% CI), [&]Fisher's Exact Test

In this study, when NOS3 (894 G>T) and NOS3 Intron 4 VNTR (894 G>T and Intron 4 VNTR of NOS3) are evaluated in CML patients, there is no observation of deviations in the NOS3 (intron 4 VNTR) polymorphism in CML as the control and CML groups were compared in terms of Hardy-Weinberg Equilibrium (HWE); however, there was a deviation (p: 0.033) in the control group.

Also, there is no observation of deviations in the NOS3 (*894 G>T*) polymorphism in the control group. When the CML and the control groups have been compared in terms of HWE; in the CML patient group, a deviation has been detected (p: 0.015). In terms of both genotype and allele frequencies when the two variants were analyzed, the researchers detected an important association.

Nitric oxide is produced by endothelial nitric oxide synthase (eNOS or NOS3) and in different processes of carcinogenesis, NOS3 genetic variant has a significant role. The current studies about the relationship between NOS3 894 G>T and NOS3 intron 4 (4A/B) polymorphisms and cancer risk demonstrate that the results are inconclusive and conflicting. However, it is suggested by a meta-analysis that there is no association between the changing risk of overall cancer and G894T and 4A/B variants of the NOS3 gene (Haque et al. 2015). He et al. (2014) claim that the NOS3 894 G>T variant might be a biomarker in order to predict overall survival (OS) in Chinese patients with AML.

A higher TT genotype of eNOS G894T in CLL patients was observed by Pehlivan et al. (2017). AlFadhli (2013) was the first to reveal an important relationship between the 4bb genotype of the 27-bp VNTR and susceptibility to Hashimotos thyroiditis (HT). The number of 27-bp repeats is in relation with the expression of NOS3 as heterozygous 4bb repeats show a decrease in NOS3 expression.

CONCLUSION

In this study, in a Turkish cohort, the relationship between G894T and intron 4 A/B variants was examined for susceptibility to CML. To summarize, two variants of the NOS3 gene in CML patients were first analyzed simultaneously in this study and it was demonstrated that these polymorphisms could play a role in the etiopathogenesis of CML.

RECOMMENDATIONS

The NO gene related molecules in CML and the role of the NO gene should be studied further.

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