RESEARCH ARTICLE

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TNF-α-308A allele Carrier Induced to Development of Chronic Lymphocytic Leukemia in Sudanese Population at Earlier Age

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Abstract

Background: several studies have been performed to investigate the association of *TNF-α*-308G>ASNP and CLL susceptibility However, the results are inconsistent. This study aimed to investigate the association between *TNF-α*-308G>ASNP of the *TNF-α* gene and CLL risk in the Sudanese population and correlated genotypes with clinicopathological features. **Methods:** A case-control study was conducted in Khartoum state, during the period from April 2017 to April 2018, involved 110 CLL patients and 50 healthy volunteers. Physical examination, Complete Blood Count, and immunophenotype were performed in all patients to confirm the diagnosis. Clinical staging such as Rai and Binet were studied. CD38 and ZAP70 were performed by Flow Cytometry. Blood samples were collected from all participants; DNA was extracted by using ANALYTIKJENA Blood DNA Extraction Kit and analyzed *TNF-α*-308G>ASNP by using AS-PCR. The statistical analysis was performed using SPSS. **Results:** *TNF-α*-308G>A genotype frequencies were GG (10.0%), GA (87.3%), and AA (2.7%) among the CLL patients, and GG (14.0%), GA (80.0%), and AA (6.0%) in the control group. The comparison of CLL patients with the control group did not show any statistically significant relationship for the genotypic and allelic frequencies. Furthermore, no association was observed between the *TNF-α*-308G>ASNP and gender, hematological parameters, clinical stages systems, CD38 expression, and ZAP-70 expression. The presence of the*TNF-α*-308Aallele was associated with a lower mean age. **Conclusions:** These results indicate that *TNF-α*-308G>A genotypes are not involved in the predisposition to the development of CLL. *TNF-α*-308A allele carrier induced to development of CLL at an earlier age.

Keywords: *TNF-α*-308G>A- (rs1800629)- CLL- clinical- hematological- markers- susceptibility- Sudan

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Introduction

Chronic lymphocytic leukemia is a chronic lymphoproliferative malignancy characterized by progressive lymphocytosis caused by the clonal accumulation of B-cells in peripheral blood, bone marrow, and lymphoid organs (Swerdlow et al., 2016). Many pleiotropic proinflammatory cytokines play a role in the activation, growth, and apoptosis of leukemic B-cells (Cordingley et al., 1988). Tumor Necrosis Factor–α is one of the most important pro-inflammatory and tumor-related cytokines for regulating immune response, inflammation, Th1/Th2 balance, and lymphomagenesis (Sethi et al., 2008). B-CLL cells can produce TNF, which is known to stimulate their proliferation in an autocrine and paracrine manner (Cordingley et al., 1988). *TNF-α* increases the proliferation and growth of CLL cells, but it may also induce cell apoptosis and necrosis (Younes and Aggarwall, 2003).

The *TNF-α* gene is located in the short arm of chromosome 6p21.3, in the class III region of the major histocompatibility complex, and consists of four exons and three introns (Makhatadze, 1998). *TNF-α* production is generally regulated at the transcriptional level (Raabe et al., 1998).

Several single nucleotide polymorphisms of the *TNF-α* gene have been described inside the *TNF-α* promoter positioned (Elahi et al., 2009). Among the various SNPs of *TNF-α*, the -308G>A polymorphism; exchange of guanine by adenine at position -308 of the

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TNF promoter region is associated with higher serum levels of soluble TNF (Wilson et al., 1997). *TNF-α*-308A allele is associated with higher constitutive and inducible *TNF-α* expression by affecting a consensus binding site of a transcription factor named activator protein-2 (AP-2) (Kroeger et al., 1997). Approximately 60% to 70% of the white population are homozygous for the wild-type TNF1 allele, 30% to 40% are heterozygous, and 1.5% to 3% are homozygous for the variant TNF2 allele (Wihlborg et al., 1999).

To date, several studies have reported that *TNF-α-308G>A* gene polymorphism is associated with susceptibility to different cancer types such as Cervical Cancer (Hamadani et al., 2017) and CLL (Demeter et al., 1997; Wihlborg et al., 1999; Mainou-Fowler et al., 2000; Au et al., 2006; Bogunia-Kubik et al., 2006; Cerhan et al., 2008; Ennas et al., 2008; Jevtovic-Stoimenov et al., 2008; Bakirov et al., 2009; Abdou et al., 2010; Fernberg et al., 2010; Lech-Maranda et al., 2010; Bakirov et al., 2012; Lech-Maranda et al., 2013; Jevtovic-Stoimenov et al., 2017; Ovsepyan et al., 2017). However, the results are inconsistent and controversial because of the small sample size of individual study, the ethnic origin of the patients, and possibly because of CLL heterogeneity. These findings indicate that there remains a great controversy regarding the association between *TNF-α*-308G>A polymorphism and CLL risk.

To the best of the authors' knowledge, no published studies are describing the possible association between *TNF-α-308G>A* gene polymorphism and CLL susceptibility in the Sudanese population. This study aimed to investigate the association between *TNF-α*-308G>A SNP of the *TNF-α* gene and CLL risk in the Sudanese population. We also examined the correlation between this SNP and CLL clinical parameters as well as hematological profile and some poor prognostic markers such as CD38 expression and ZAP-70 expression.

Materials and Methods

Study Population

This study is a case-control study, conducted in Khartoum state, Sudan, in the period from April 2017 to April 2018, 110 patients with CLL and 50 healthy volunteers as a control group were recruited to participate in this study. Patients were obtained at Flow Cytometry Laboratory for Leukemia & Lymphoma Diagnosis, Khartoum; they were referred for immunophenotype diagnosis.

All patients were diagnosed based on clinical history, physical examination, and complete blood count. The peripheral blood is important to show morphological abnormalities and immunophenotypic criteria. All our patients have $\geq 5{,}000 \times 10^9$ /L B lymphocyte, considered in our diagnosis according to the International Workshop on Chronic Lymphocytic Leukemia (Hallek et al., 2008). The stage of the CLL was assessed by Rai and Binet classification (Rai et al., 1975; Binet et al., 1981). All patients were newly diagnosed without any previous treatment, whereas patients with other lymphoid neoplasms (both B and T-cell Lineage) were excluded.

Sample Collection

The peripheral blood was collected as samples from both groups included in the study (the patient group and control group).For the patient group, an amount of four milliliters (ml) of blood was collected from each patient in (EDTA) and divided equally into two tubes; one tube for complete blood count and immunophenotype test and the other tube for molecular analysis. For the control group, two (ml) of blood was collected from each healthy individual in (EDTA) for molecular analysis.

Determination of Blood Count

Two ml of peripheral blood were withdrawn from each patient; these samples were collected in EDTA tubes and preserved at room temperature (22-24^{o}C) then processed within 6-24h from the collection. Complete blood count was analyzed by using automated hematology analyzer (SYSMEX KX-21N, Japan). All results such total WBC, Absolute lymphocyte count, Hemoglobin level, RBC, and platelets were recorded.

Determination of Immunophenotyping and (CD38 & ZAP-70 expression)

The diagnosis of CLL was confirmed in each patient by Flowcytometry (EPICS XL Beckman Coulter Flow Cytometry, Miami, FL, USA), standard protocol of Beckman Coulter was used in fluorescent dye-labelled monoclonal antibody for CD45, CD3 CD5, CD10, CD19, CD20, CD22, CD23, FMC7, CD79b, kappa, and lambda light chain. A marker was considered positive at cutoff ≥30%. However, in order to confirm diagnostic CLL, a scoring system was applied depending on Moreau et al., (1997). Absolute B lymphocyte count was obtained by Flow Cytometry and ZAP-70 and CD38 were used as prognostic markers, with a cutoff point of 20% and 30%, respectively, as previously described (Basabaeen et al., 2019).

DNA Extraction

After confirmed immunophenotyping of patients, genomic DNA was extracted from all blood samples of patients and control groups by using ANALYTIKJENA Blood DNA Extraction Kit (Germany) (REF-845-KS-1020050), according to the manufacturer's instructions. The β-globin gene was used to assess the quality of DNA in all extracted samples, the primer sequences of β globin as previously described (Kerr et al., 2000). All specimens for β-globin gene were Successful amplification with product size 268bp. To evaluate the DNA quantification after DNA extraction, we measured DNA by using a NanoDrop spectrophotometer. Then DNA samples were routinely at -20^{o}C.

Determination of TNF-α-308G>A rs1800629 Genotype by Allele Specific-Polymerase Chain Reaction

Detection of the *TNF-α*-308G>A polymorphism based upon the method of Kamali-Sarvestani et al, three primers were used for AS-PCR was used to detect the polymorphism at position -308 of *TNF-α* the following primers were used, G allele: forward (5'ATAGGTTTTGAGGGGCATGG-3'), common reverse

(5'- TCTCGGTTTCTTCTCCATCG -3') *TNF-α* Aallele: forward (5'-ATAGGTTTTGAGGGGCATGA-3') (Kamali-Sarvestani et al., 2005).

Two separated PCR reaction mixtures of 20µl were prepared for each sample. PCR was performed by using 4µl 5× HOT FIREPol Blend Master Mix, (Solis BioDyne,Estonia), Cat. No. 04-25-00125), 2µl of genomic DNA, 0.5 µl of each primer, and 13 µl distilled water. Thermocycling conditions for both alleles include initial denaturation at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57.2°C for 30 seconds, and extension at 72°Cfor 20 seconds, followed by a final extension at 72°C for 5 minutes. Thermocycling was using TECHNE Tc-412-UK PCR Thermal Cycler 96 well. After amplification, PCR products and 100 bp DNA ladder (iNtRON BIOTECHNOLOGY, KOREA), were run on 2% agarose gel containing ethidium bromide and identified under UV transilluminator using gel documentation system (SYNGENE, JAPAN) (Figure 1).

Statistical Analysis

Data was analyzed using the statistical package for social sciences version 23.0 (Chicago, IL, USA). Numerical data were summarized as mean and standard deviation and n (%) of study participants, respectively. Logistic regression was used for the calculation of odds ratio with a confidence interval for risk estimation. The Hardy-Weinberg equilibrium was tested by the goodness of fit X2 test to compare the observed genotypic frequencies in normal individual to the expected genotypic frequencies, and then calculated from the observed allelic frequencies. A Chi-Square test was used for analyzing associations between categorical variables. One-Way ANOVA was used to compare the means of two groups. All P-values were two-sided, and <0.05 was considered as the significance level.

Results

The mean age was 62.97 (range: 22-85 years) for patients and 63.22 (range: 24–83 years) for controls, and male participants accounted for a greater proportion in both groups (71.8% and 72.0%, respectively). No significant differences were found in age and gender between cases and controls (age: p 0.900; sex: p 0.981); therefore, the patients and controls seemed to be adequately matched in terms of age and gender.

The *TNF-α*-308G>A genotype frequencies were GG (10.0%), GA (87.3%), and AA (2.7%) among the CLL patients, and GG (14.0%), GA (80.0%), and AA (6.0%) in the control subjects. The comparison of patients with CLL with the control group did not show any statistically significant relationship for the genotypic and allelic

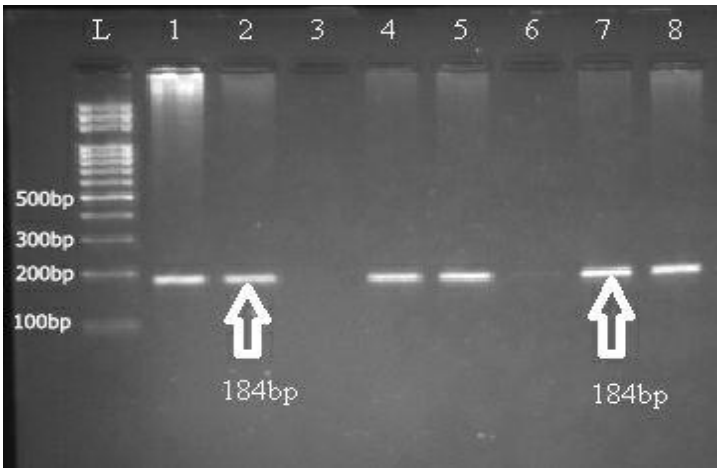

Figure 1. *TNF-α*-308G>A Genotyping Using AS-PCR. Lane L, 100 bp DNA molecular weight marker. Lanes 1, 5, and 7, allele G is represented by the presence of a 184bp PCR fragment. Lanes 2, 4, and 8, allele A is represented by the presence of a 184bp PCR fragment. Each two lanes represent one sample, lanes 1 and 2, heterozygous (GA); lanes 3 and 4, homozygous (AA); lanes 5 and 6, homozygous (GG); lanes 7 and 8, heterozygous (GA).

Table 1. Frequency of the *TNF-α* -308G>A Genotype in Patients with Chronic Lymphocytic Leukemia and Control Group

Allele / Genotype	CLL group (N=110) (%)	Control group (N=50) (%)	OR (95% CI)	P- value
GG	11/110 (10.0)	7/50 (14.0)	0.68 (0.25-1.88)	0.457
GA	96/110 (87.3)	40/50 (80.0)	1.71 (0.70-4.18)	0.232
AA	3/110 (2.7)	3/50 (6.0)	0.44 (0.09-2.26)	0.312
GA+AA	99/110 (90.0)	43/50 (86.0)	1.47 (0.53-4.03)	0.457
G allele frequency	118 (53.6)	54 (54.0)	0.99 (0.61-1.58)	0.951
A allele frequency	102(46.4)	46 (46.0)	1.01 (0.63-1.63)	
HWE: P *	0.001	0.001		

OR, odds ratio; 95% CI, 95% confidence interval; P*, significance probability for deviation from Hardy-Weinberg equilibrium (HWE), P value significant below 0.05.

Table 2. Associations between *TNF-α* -308G>A Genotype and CLL Prognostic Markers

Prognostic marker	Genotype			P- value
	GG	GA	AA	
No. of Cases	11	96	3	0.431[a]
HWE	40.2	52.6	17.2	
Mean Age	66.27y	63.26y	41.67y	0.005[b]
Sex (%)				
Male	6 (54.54)	71 (74.0)	2 (66.66)	0.391[c]
Female	5 (45.45)	25 (26.0)	1 (33.33)	
Binet Stage (%)				
A	3 (27.27)	28 (29.16)	2 (66.66)	0.596[c]
B	3 (27.27)	31 (32.29)	1 (33.33)	
C	5 (45.45)	37 (38.54)	0 (0.0)	
Rai Stage (%)				
0	1 (9.1)	8 (8.33)	1 (33.33)	0.099[c]
I	1 (9.1)	21 (21.87)	1 (33.33)	
II	3 (27.3)	19 (19.79)	1 (33.33)	
III	1 (9.1)	35 (36.45)	0 (0.0)	
IV	5 (45.5)	13 (13.54)	0 (0.0)	
CD38 (%)				
<30%	5 (45.45)	62 (64.58)	2 (66.66)	0.457[c]
≥30%	6 (54.54)	34 (35.41)	1 (33.33)	
ZAP70 (%)				
<20%	5 (45.45)	66 (68.75)	3 (100.0)	0.140[c]
≥20%	6 (54.54)	30 (31.25)	0 (0.0)	

a, compared to the control group; b, ANOVA test; c, Chi Square test. (n=110), P value significant below 0.05.

Table 3. Associations between *TNF-α* -308G>A Genotype and Hematological Parameters

Parameter	Genotype						P. value
	GG		GA		AA		
	No.11		No.96		No.3		
	Mean	SD	Mean	SD	Mean	SD	
WBC×10^3/ul	124.2	83.27	89.89	75.18	72.96	23.81	0.327
Absolute- Lymphocyte×10^3/ul	116.07	79.94	79.06	70.16	59.65	21.87	0.224
Absolute B Lymphocyte×10^3/ul	107.77	78.48	72.12	67.3	52.72	21.61	0.221
RBC×10^6/ul	3.41	0.91	3.71	0.94	4.57	0.21	0.164
Lymphocyte %	91.45	5.44	84.46	8.81	81	3.6	0.027
Platelets×10^3/ul	143.72	103.31	191.15	100.8	295	188.39	0.075
Hemoglobin (g/dl)	10.37	2.35	11.16	2.47	13.53	0.55	0.143

ANOVA test; (n, 110). P value significant below 0.05

frequencies. Genotype frequencies from this case-control study are shown in (Tables 1, 2). Further, the genotype frequencies for *TNF-α*-308G>A SNP among both the case and control groups were found to be in disagreement with Hardy-Weinberg equilibrium (HWE) (Table 1). Frequency heterozygote GA was higher than that for homozygote *TNF-α* (-308GG &-308AA) in patients and control group.

The mean ages at diagnosis differ between the three groups: 66.27, 63.26, and 41.67 years in patients with -308GG, -308GA, and -308AA genotypes, respectively. This study found that there was a significant difference for mean age according to genotype frequencies in the case group (Table 2).

We were unable to identify significant differences according to gender, Rai stage, and Binet stage system as well as hematological parameters between patients with variant genotypes of the studied polymorphism (Tables 2, 3).

We divided patients into the following groups (using a 30 % cutoff value for CD38 and 20 % cutoff value for ZAP-70). Next, we compared the genotype distribution of *TNF-α*-308G>A between CD38 and ZAP-70 in CLL patients, but we did not find any significant differences (Table 2).

Discussion

Recently, several gene polymorphisms have been suggested to correlate with clinical outcome in CLL. There are conflicting data about the association of *TNF-α*-308G>A polymorphism and raised risk of CLL.

In the present study, we did not find any significant association for the genotype nor the allelic frequency in patients with CLL regarding the *TNF-α*-308G>A polymorphism. Also, dichotomizing by dividing the *TNF-α*-308G>A genotypes as A+ genotypes (combining the GA and AA genotypes as one group) and G genotype (GG) also failed to indicate any significant associated with an increased risk of CLL development. In a similar result, most previous studies did not find any relationship between risk of CLL and the *TNF-α*-308G>A genotype frequency (Wihlborg et al., 1999; Mainou-Fowler et al., 2000; Au et al., 2006; Bogunia-Kubik et al., 2006; Cerhan et al., 2008; Ennas et al., 2008; Jevtovic-Stoimenov et al., 2008; Abdou et al., 2010; Fernberg et al., 2010; Lech-Maranda et al., 2010; Lech-Maranda et al., 2013).

However, some studies found a significant increase in the frequency of the GG Genotypes and G allele in a polymorphic locus-308G>A gene *TNF-α* has appeared as markers of the raised risk in CLL development (Demeter et al., 1997; Bakirov et al., 2009; Bakirov et al., 2012).

In contrast, Ovsepyan et al. and Jevtovic-Stoimenov et al. suggested that the presence of *TNF-α*-308A allele (-308AG & -308AA) may be associated with an increased risk of CLL development (Jevtovic-Stoimenov et al., 2017; Ovsepyan et al., 2017).

Zhai et al., (2014) were performed a meta-analysis study to assess the effect of *TNF-α*-308G>A polymorphism on the risk of NHL and various subtypes in Caucasian and Asian populations, this meta-analysis study indicated that *TNF-α*-308G>A polymorphism is not associated with CLL risk in overall and each ethnic subgroup. No available studies in the literature describing the possible impact of *TNF-α*-308G>A polymorphism on susceptibility to CLL development in the African population, especially in the Sudanese population.

Surprising the distribution of *TNF-α*-308G>A genotypes among the healthy controls in our study is dissimilar to those obtained in the previous study conducted in healthy unrelated individuals in Sudan (Bereir et al., 2003). In the present study, the genotype distribution of *TNF-α*-308G>A polymorphism had shown significant (HWE $p<0.001$) deviation in both controls and CLL cases from the Hardy–Weinberg equilibrium. Similar to a previous study conducted in Sudan that included four Sudanese populations of different geographic origins (Bereir et al., 2003). This study suggested *TNF-α*-308G>A SNP departure from HWE in One population namely Nilotics (Bereir et al., 2003). This indicated inter-population variation for genotype frequencies at this locus (Bereir et al., 2003).

Reports have demonstrated that the *TNF-α* -308GG genotype frequency ranged in between 43.6% to 89.0%, while *TNF-α* -308AA (*TNF-α* -308GA or AA) genotype is present in 0.0% to 55.1% among patients with CLL (Demeter et al., 1997; Wihlborg et al., 1999; Mainou-Fowler et al., 2000; Au et al., 2006; Bogunia-Kubik et al., 2006; Cerhan et al., 2008; Ennas et al., 2008; Jevtovic-Stoimenov et al., 2008; Bakirov et al., 2009; Abdou et al., 2010; Fernberg et al., 2010; Lech-Maranda et al., 2010; Bakirov et al., 2012; Lech-Maranda et al., 2013; Jevtovic-Stoimenov et al., 2017; Ovsepyan et al., 2017). No available studies in the literature describing the frequency of *TNF-α*-308G>A polymorphism among patients with CLL in the African population, especially in the Sudanese population. The present study showed a higher frequency of *TNF-α* -308GA heterozygote in patients and control group. Also some studies performed in Egypt and Northern Ivory Coast demonstrated a higher frequency of the *TNF-α*-308GA genotype 86.7% and 60.0%, respectively(Settin et al., 2007; Santovito et al., 2012). The variations found in the literature compared to those obtained from our study could be justified by the high heterogeneity degree seen in the African population. This observation suggests that the distribution of this polymorphism varies because of ethnic factors.

In our study, the presence of the*TNF-α*-308A allele (*TNF-α* -308GA or AA) was associated with a lower mean age. In fact, most studies have failed to show a statistically significant correlation between age and *TNF-α*-308G>A polymorphism in CLL patients (Wihlborg et al., 1999; Au et al., 2006; Jevtovic-Stoimenov et al., 2008; Lech-Maranda et al., 2010; Lech-Maranda et al., 2013). Interestingly, Wihlborg et al., (1999) showed the mean age at diagnosis of Hodgkin's disease patients differed between the groups and were 36.5 and 28.2 years for *TNF-α*-308GG and *TNF-α*-308GA, respectively. This indicated *TNF-α*-308A allele carrier induced to development of lymphoproliferative disorders at earlier age. Since no reports of the *TNF-a* gene polymorphism in CLL exist in the literature, this is a finding requiring further study.

In the present study, the gender ratio was 2.5:1 men for each woman in patients with CLL. We did not find any significant relationship for the genotype distribution and gender. In a similar result,Jevtovic-Stoimenov et al. and Lech-Maranda et al. did not find any relationship between gender and the *TNF-α*-308G>A genotypes distributions (Jevtovic-Stoimenov et al., 2008; Lech-Maranda et al., 2013). On contrast, a study conducted in the Chinese population found *TNF-α* -308A allele is significantly associated with female CLL cases (Au et al., 2006). Moreover, another study conducted in the Serbian population suggested that *TNF-α*-308A allele associated with male subjects with CLL (Jevtovic-Stoimenov et al., 2017). We are not able to provide an explanation of this observation at this moment, but the variation of male to female ratio and ethnicity heterogeneity cannot be excluded.

In this report, we note no significant association was observed with the *TNF-α* -308G>A genotypes and Rai stage system. A previous studies also found no association between the presence of *TNF-α*-308G>A polymorphism and Rai stage system (Demeter et al., 1997; Mainou-Fowler et al., 2000; Bogunia-Kubik et al., 2006; Jevtovic-Stoimenov et al., 2008; Lech-Maranda et al., 2010; Lech-Maranda et al., 2013).

In this study no significant difference was found in

TNF-α-308G>A genotype distributions in relation to Binet system. Regarding the Binet stage system, findings of this study are in line with previous studies observations in CLL patients (Demeter et al., 1997; Mainou-Fowler et al., 2000; Bogunia-Kubik et al., 2006; Jevtovic-Stoimenov et al., 2008; Ovsepyan et al., 2017). It should be noted there was no indication that the*TNF-α*-308G>A polymorphism affects disease progression in CLL patients.

In the present study statistical analysis did not reveal any differences in hematological parameters according to *TNF-α*-308G>A genotypes frequencies. In a similar study by AU et al. did not appear to be a relationship between *TNF-α*-308G>A genotype distributions and white cell count at diagnosis (Au et al., 2006). No another study found on literature discussed such this association.

Our study, did not find any significant association in CD38 expression as well as ZAP-70 expression between CLL samples displaying the various *TNF-α*-308G>A genotypes. These results are in keeping with previous findings in other reports (Lech-Maranda et al., 2010; Lech-Maranda et al., 2013), confirming he *TNF-α*-308G>A polymorphism was not associated with any prognostic markers such as CD38 and ZAP-70 expression CLL patients. In contrast Polzonetti et al. suggested *TNF-α*-308G>A was significantly associated with CD38 activity in healthy individuals by using an RP-HPLC method (Polzonetti et al., 2012).

In conclusion, the frequency of the *TNF-α*-308G>A promoter polymorphism was not significantly different between CLL patients and compared to controls. These results indicate that *TNF-α*-308G>A) alleles are not involved in the predisposition to the development of CLL. Our study showed a higher frequency of *TNF-α* -308GA heterozygote in patients and control group. The presence of *TNF-α*-308A allele (*TNF-α* -308GA or AA) was associated with lower mean age. No association was shown between the *TNF-α*-308G>A genotypes and gender, hematological parameters, and clinical stages systems as well as CD38 expression or ZAP-70 expression.

Author Contribution Statement

AAB & EAA & IKI conceived the study design, participated in data collection, performed the statistical analysis, interpreted the results, and revised the manuscript. EAB, NMA, SOA & AYA participated in the statistical analysis and drafted the manuscript. OAA and EAF participated in the data collection, carried out the laboratory work, and prepared the results.AAB performed molecular analysis. All authors read and approved the final manuscript.

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Availability of data and materials

The individual data are available in the archives of the Flow Cytometry for Leukemia & Lymphoma Diagnosis, Khartoum, Sudan and can be obtained from the corresponding author on request.

Consent for publication

Not applicable

Ethics approval and consent to participate

Ethical clearance was obtained from the Institutional Review Board at Al Neelain University. The principal investigator obtained written informed consent from all participants prior to their inclusion in the study.

Conflict of interest

The authors declare that they have no conflict interests.

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