Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

TNF-α -308 G>A and IL10 -1082A>G polymorphisms as potential risk factors for lymphoproliferative disorders in autoimmune rheumatic diseases

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Abstract

Background: Chronic inflammation with sustained unregulated immune stimulation in autoimmune rheumatic diseases (ARD) may be a risk factor for developing lymphoproliferative disorders (LPD). Markers of ARD activity as high erythrocyte sedimentation rate or erosive joint diseases and the development of B-symptoms were accounted as risk factors for LPD development. We investigated the association of five inflammatory cytokine genes single nucleotide polymorphisms (SNPs): TNF-α -308G>A; TGF-β1 gene codon 10 T>C and 25 G>C; IL-10 promoter SNPs -1082 A>G, -819T>C, and -592A>C; IL-6 -174G>C; and IFN-γ 874 T>A with the risk of LPD development in ARD patients. The study was conducted on 70 patients divided into group I, 25 ARD patients diagnosed as RA (n = 15) and SLE (n = 10) and with no history of malignancy; group II, 25 patients diagnosed with LPD and had no ARD; and group III, 20 patients diagnosed with both diseases: ARD and LPD. Cytokine genotyping was analyzed by PCR-sequence-specific primer (PCR-SSP).

Results: ARD+LPD patients had significantly higher frequency of TNF-α -308A allele and AA+AG genotype (high TNF-α producers) and IL-10 -1082A allele and AA genotype (low IL-10 producers) than ARD patients (p = 0.003, p = 0.024, p = 0.003, p = 0.03, respectively) with a significantly increased risk of LPD development in ARD patients expressing the corresponding alleles and genotypes. No significant differences were detected in the distribution frequency of either TGF-β1, IL-6, or IFN-γ SNPs between groups I and III or any of the studied SNPs between groups II and III. The distribution frequency of IL-10 ATA haplotype was significantly increased in group III as compared to group I (p = 0.037).

Conclusion: The significantly increased frequency of the high-TNF-α- and low-IL-10-producing alleles and genotypes in ARD patients may participate in the provision of a proinflammatory milieu that eventually increases the risk of LPD development.

Keywords: Autoimmune rheumatic disease (ARD), Cytokines, Lymphoproliferative diseases (LPD), Single nucleotide polymorphisms (SNPs), PCR-sequence-specific primer (PCR-SSP)

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Background

Autoimmune rheumatic diseases (ARD) are heterogeneous immune disorders characterized by a dysregulated immune response and chronic inflammation [1]. ARD include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), systemic sclerosis (SSc), polymyositis (PM), and dermatomyositis (DM) [1]. ARD patients are at an increased risk of developing lymphoproliferative disorders (LPD) such as non-Hodgkin (NHL) and Hodgkin (HL) lymphoma than the general population [2–8]. Diagnosis of malignancies is a major challenge in ARD patients, where some patients may develop malignancies that mimic the initial rheumatic clinical symptoms, hence complicating the process of distinguishing malignancy from aggravation of the initial disease [6]. Nevertheless, it is believed that the rheumatic symptoms may bring malignancies to clinical consideration earlier [6].

Several hypotheses were suggested to elucidate lymphomagenesis process in ARD including the genetic predisposition [9, 10], dysregulated immune stimulation [11, 12], detrimental effect of immune-suppressive therapy [3, 13], and environmental factors [7, 14]. Genetic predisposition linking ARD and LPD requires further comprehensive studies, as the overall occurrence of lymphomas in first-degree relatives of RA patients did not significantly differ from that anticipated in the general population [9, 10]. Meanwhile, ARD treatment protocols', such as immunosuppressive drugs or disease-modifying antirheumatic drugs (DMARD), role to increase LPD risk in ARD could not be confirmed [3, 13].

Dysregulated immune stimulation and chronic inflammation are the most noteworthy studied risk for lymphomagenesis in ARD patients. Information on the disease characteristics along with the general reviewing of the systems in ARD patients can unveil the development of some worrisome signs that raise the suspicion of LPD development, e.g., high ESR values, erosive joint diseases, lymph node enlargement, organomegaly, night sweating, fever, and weight loss may alarm the development of malignancy [15–17]. It is suggested that pathogenesis of ARD that resulted from chronic B cell activation and inflammation are risk determinants of malignant transformation of B cells and lymphoma development. Plentiful data have been presented on the role played by cytokines in the pathogenesis of both ARD and LPD; however, studies still demonstrate the functional and the prognostic potentials of cytokines at cellular and molecular levels [11, 18, 19].

Several attempts have been tried to define diagnostic biomarkers to stratify ARD patients who are at high risk of LPD using serological immune markers such as cytokines, chemokines, paraproteins, and soluble receptors or genetic biomarkers such as cytokine gene SNPs. A study was conducted by England et al. to evaluate the association of 17 circulating cytokines and chemokines with cancer mortality in RA men patients, where LPD was the second commonest detected cancer [12]. The study revealed that patients with the highest quartile level of 7 cytokines: interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, and macrophage inflammatory protein 1β (MIP-1β), had a > 2-fold increased risk of overall cancer mortality [12]. Makgoeng et al. provided evidence that the elevated expression of immuno-inflammatory markers such as tumor necrosis factor- alpha (TNF-α), CXCL13, sCD23, sCD27, and sCD30 was associated with an increased risk of NHL [20]. An interesting study conducted on primary Sjogren's syndrome showed that low complement factors C3 and C4, and $CD4^+$ cytopenia with a low $CD4^+/CD8^+$ T cell ratio were predictors of LPD development [21].

Hence, it is of scientific and clinical necessity to identify ARD patients who are at an increased risk of developing LPD. The current study aimed to evaluate the cytokine SNPs in TNF-α promoter, transforming growth factor-β (TGF-β1) gene codon, interleukin-10 (IL-10) promoter, IL-6 promoter, and interferon-gamma (IFN-γ) genes as possible risk factors for lymphomagenesis in ARD patients. A conjoint study of the pro- and anti-inflammatory cytokine genes polymorphisms may help in understanding the disease pathogenesis, hence implementing novel diagnostic genetic markers.

Methods

Subjects

The present study was conducted on 70 patients recruited from the Rheumatology and Hematology Units and the Outpatient Clinics, main University Hospital. The patients were divided into three groups: group I and II representing the controls and group III representing the cases. Group I has 25 ARD patients, including RA (n = 15) and SLE (n = 10) and with no recorded history of previous malignancy. ARD patients were diagnosed according to the American College of Rheumatology, and the disease activity was assessed using the Disease Activity Score 28 (DAS28) for RA and BILAG-2004 index for SLE [22, 23]. Group II has 25 patients diagnosed with LPD and included chronic lymphocytic leukemia (CLL), n = 10; non-Hodgkin lymphoma (NHL), n = 8; and Hodgkin lymphoma (HL) n = 7 and with no diagnosed systemic rheumatologic disease. Group III has 20 patients diagnosed with both ARD+LPD and included RA+NHL, n = 7; RA+CLL, n = 2; SLE+CLL, n = 4; SLE+NHL, n = 5; and SLE+Castleman disease, n = 2. The study was conducted after the approval of the Ethics Committee of the University and in accordance with the Declaration of Helsinki (reference number: 020753). Written informed consents were obtained from the studied subjects. Exclusion criteria were a previous diagnosis or treatment from malignancy other than LPD, history

of HCV or HIV infection, previous exposure to therapeutic irradiation, and pregnant females.

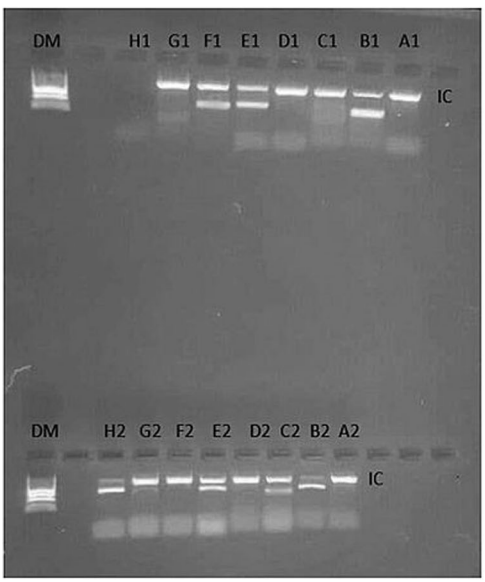
Laboratory investigations done were complete blood count (CBC) and immunological tests to confirm the diagnosis of the rheumatologic diseases such as anti-nuclear Ab (ANA), anti-double-stranded DNA (anti-dsDNA), rheumatoid factor (RF), and anti-cyclic citrullinated protein (ant-CCP). Inflammatory markers such as ESR first hour (mm), C-reactive protein (CRP) (mg/dl), serum ferritin (ng/ml), serum lactate dehydrogenase (LDH) (U/L), and β2-microglobulin (β2-M) (mg/l). Coomb's test (anti-globulin test) was done for autoimmune hemolytic anemia (AIHA) diagnosis. Bone marrow aspiration, trephine biopsy, and immunophenotyping were used for LPD diagnosis.

Cytokine genotyping by PCR-sequence-specific primer (Fig. 1)

Genomic DNA was extracted from peripheral blood leukocytes using Quick-DNA Miniprep #Kit D3024 (ZYMO Research CORP, CA, USA) following the manufacturer's instructions. Cytokine genotyping by PCR-sequence-specific primer (PCR-SSP) amplification was done for five cytokine genes SNPs including: TNF-α promoter -308G>A (rs1800629); two TGF-β1 SNPs, codon 10 (+869) C>T (rs1 982073 merged into rs1800470) and codon 25 (+915) G>C (rs1800471); three IL-10 promoter SNPs, -1082A>G (rs18 00896), -819 T>C (rs1800871), and -592A>C (rs1800872); IL-6 promoter -174C>G (rs1800795); and IFN-γ +874T>A (rs2430561). Human β-globin gene was used as an internal control. Cytokine genotyping was done using Cytokine Genotyping Tray (One Lambda, Canoga Park, CA) and carried out according to the manufacturer's recommendations. PCR amplification protocol was as follows: 1 cycle for 130 s at 96°C followed by 60 s at 63°C; then 9 cycles of 10 s at 96°C followed by 60 s at 63°C; then final 20 cycles of three different temperature ramps included denaturation for 10 s at 96°C, annealing for 50 s at 59°C, and extension for 30 s at 72°C. The PCR amplicon bands were visualized by UV transilluminator for gel interpretation. A positive reaction for a specific cytokine gene SNP was detected as a band between the internal control and the primer dimers band.

Statistical analysis

Statistical analysis was done using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Qualitative data were analyzed using the chi-square test and the Monte Carlo test. Normally distributed quantitative data were analyzed using the Student t test. Significant p value was assumed at ≤ 0.05. Odds ratios (ORs) are given with 95% confidence intervals (CI). ORs were performed for the allele model where the major allele (M) and minor allele (m) study assessed the impact of individual allele on the disease. For the statistical significant SNPs, the following models were analyzed: the homozygote model MM vs mm, the heterozygote model Mm vs MM, the dominant model MM vs Mm+mm, the over



Lane	Allele represented
A1	IL-10 (-1082A, -819T)
B1	TGF-β1 codon 25 "G"
C1	TGF-β1 codon 25 "C"
D1	TGF-β1 codon 10 "C"
E1	TGF-β1 codon 10 "T"
F1	TNF-α promoter (-308G)
G1	TNF-α promoter (-308A)
H1	Negative Control

Lane	Allele represented
A2	IFN-γ (+ 874A)
B2	IFN-γ (+ 874T)
C2	IL-6 (-174G)
D2	IL-6 (-174C)
E2	IL-10 (-819C, -592C)
F2	IL-10 (-819T, -592A)
G2	IL-10 (-1082A, -819C)
H2	IL-10 (-1082G, -819C)

Fig. 1 Cytokine genes SNPs genotyping by PCR-SSP. Five cytokine genes SNPs including TNF-α promoter -308G>A, two TGF-β1 SNPs; codon 10 C>T and codon 25 G>C; three IL-10 promoter SNPs;-1082A>G, -819T>C, and -592A>C; and IL-6 promoter -174C>G and IFN-γ +874T>A were genotyped by PCR-SSP (described in methodology). The SNP bands were visualized by 3% agarose gel electrophoresis. IC: internal control used was human β-globin gene. DM: 100 bps DNA marker. The IC and primer dimer bands served as size markers. The band between the two size markers was the positive typing band. The lanes were marked alphabetically, and the corresponding cytokine SNP alleles are summarized in the tables (right-hand side). The figure represents a case genotyped in the present study

dominant model Mm vs MM+mm, and the recessive model mm vs MM+Mm [24]. The three IL-10 SNPs: -1082A>G, -819T>C and -592A>C generated three commonly studied haplotypes ATA, ACC, and GCC that represented three different secretion phenotypes: low, intermediate, and high IL-10 producers, respectively [25]. IL-10 haplotype frequencies and distribution study were conducted using Haploview 4.2 software.

Results

Demographic data

Demographic data of the three studied groups are presented in Table 1 and showed the mean age in group I was 44 ± 10.5 years and the mean time duration of ARD disease was 18 ± 10.2 years. In group II, the mean age was 43.3 ± 9.7 years and the mean time duration of LPD disease was 7.9 ± 1.8 years. In group III, the mean age was 49.8 ± 5 years and the mean time duration of ARD and LPD disease was 13 ± 8.9 and 3.3 ± 2.8 years, respectively. Female patients were significantly represented in the three studied groups compared to the male presentation (p = 0.017). Laboratory results for the three studied groups are enlisted in Table 2. The incidence of AIHA showed a significant difference between the three studied groups (p = 0.01), where the incidence of AIHA was significantly lower in ARD (group I) patients compared to LPDs (group II) or ARD+LPD (group III) patients.

Genotyping of cytokine polymorphism

The distribution frequency of the cytokine gene SNP alleles showed a significant difference in TNF-α -308G>A alleles between the three groups (p = 0.003) (Table 3). TNF-α -308A allele was significantly represented in group III than group I (p = 0.022), with 2.7 times elevated risk of LPD among ARD patients with TNF -308A allele (OR = 2.7, 95% CI = 1.14–6.57). TNF-α -308G allele was significantly represented in group I more than in group III with decreased risk of LPD in ARD patients with -308G allele (OR = 0.37, 95% CI = 0.15–0.88). TNF-α -308A allele was significantly represented in group II more than in group I (p = 0.001). No significant difference was detected with respect to TNF-α -308 G>A allelic distribution between groups II and III (p = 0.39).

TGF-β1 gene codon 10 and 25 polymorphism distribution showed a non-significant difference between the three groups (p = 0.57 and 0.78), respectively.

IL-10 -1082 A>G allelic frequency showed a significant difference between the three studied groups (p = 0.003). IL-10 -1082A allele was significantly represented in group III than in group I (p = 0.009), with 3.2 times elevated risk of LPD in ARD patients with -1082A allele (OR = 3.25, 95% CI = 1.31–8.04). IL-10 -1082G allele was significantly represented in group I more than in group III with decreased risk of LPD development in ARD patients with -1082G allele (OR = 0.31, 95% CI = 0.12–0.76). IL-10 -1082A allele was significantly represented in group II more than in group I (p = 0.002). No significant difference was detected between groups II and III with respect to IL-10 -1082 A>G allelic distribution (p = 0.74). No significant differences were detected between the three groups with respect to IL-10 -819 T>C or -592 A>C allelic frequency (p = 0.06, p = 0.53, respectively).

No significant differences were detected between the three studied groups with respect to IL-6 promoter or IFN-γ polymorphism allelic distribution (p = 0.06, p = 0.88 respectively).

The distribution frequency of the five cytokine SNP alleles in RA vs SLE subjects within group I and group III is represented in Table 4. The results showed that the significant differences in TNF-α -308 G>A and IL-10 -1082 A>G allelic distribution between groups I and III were detected mainly in RA subjects. TNF-α-308A was significantly represented in group III than in group I (p = 0.02), with five times increased risk of LPD in RA patients with -308A allele (OR = 5.0, 95% CI = 1.19–20.9). IL-10 -1082A was significantly represented in group III more than in group I (p = 0.009), with 5.2 times increased risk of LPD development in RA patients with -1082A allele (OR = 5.2, 95% CI = 1.44–18.7).

The study of the genetic models for the significant SNPs TNF-α -308G>A and IL-10 -1082 A>G in groups I and III is shown in Table 5. TNF-α -308 G>A genetic models showed a significant difference in the distribution frequency of the dominant model GG vs AA+GA between groups I and III (p = 0.02), which was consistent with our initial observation for -308A allele. The high TNF-α-producing phenotype associated with the -308 AA and GA genotypes [26, 27] was significantly represented in group III more than group I, with 6 times elevated risk of LPD development in ARD patients with -308AA or GA genotypes (OR = 6.0, 95% CI = 1.13–31.7).

IL-10 -1082 A>G genetic models showed a significant difference in the distribution frequency of the dominant genetic model AA vs GG+GA between groups I and III (p = 0.03), where the low IL-10 -1082AA-producing phenotype was significantly represented in group III more than in group I. The risk of LPD development was nearly four times greater in ARD patients with -1082AA genotype (OR = 3.9, 95% CI = 1.1–13.5).

Haplotype frequency of IL-10 promoter SNPs, -1082A>G,-819T>C, and -592A>C (Table 5), showed a significant increase in the distribution frequency of the low IL-10-producing ATA haplotype [26, 27] in group III compared to group I (p = 0.04), with 2.8 times increased risk of LPD development in ARD patients who were ATA haplotype carriers (OR = 2.8; CI 95% = 1.04–7.66).

No significant differences were detected for any of the studied cytokine gene polymorphism genotypes between groups II and III (data not shown).

Table 1 Demographic data of the three studied groups

	Group I (n = 25)	Group II (n = 25)	Group III (n = 20)	Test of Sig.	p
Age (yrs)					
Median (Min. – Max.)	40 (30-62)	43.5(26-58)	50 (41-55)	F =3.008	0.056
Mean ± SD.	44± 10.5	43.3± 9.7	49.8± 5		
Sex					
Male	2 (8%)	8 (32%)	9 (45%)	**χ^2=8.16***	**0.017***
Female	23 (92%)	17 (68%)	11 (55%)		
ARD					
RA	n=15	-	n= 9	-	-
SLE	n= 10	-	n=11	-	-
LPD subtypes					
HL	-	n= 7	--	-	-
B-CLL	-	n= 10	n= 6	-	-
NHL	-	n= 8	n=12	-	-
DLBCL	-	6	7	-	-
MCL	-	-	1	-	-
FL	-	1	1	-	-
T-cs lymphoma	-	1	3	-	-
Castleman's disease	-	-	n= 2	-	-
Duration of ARD (yrs)					
Median (Min. – Max.)	9 (6-18)	–	10 (2-13)	U =202.0	0.440
Mean ± SD.	18 ± 10.2	–	13 ± 8.9		
Duration of LPD (yrs)					
Median (Min. – Max.)	–	7.5 (6-12)	2 (1-10)	**U =40.0***	**<0.001***
Mean ± SD.	–	7.9 ± 1.8	3.3 ± 2.8		
Activity score					
DAS28 (for RA)	3-5	-	5.1-7		
BILAG-2004 (for SLE)	8-12		>12		
Joint affected					
Small	18(72%)	5(20%)	13(65%)	**χ^2 =15.60**	**<0.001***
Small and Large	7(28%)	20(80%)	7 (35%)		
LN enlargement	1(4%)	17(68%)	14 (70%)	**χ^2 =27.29**	**<0.001***
HSM	1(4%)	11 (44%)	7(35%)	**χ^2 =11.00**	**0.004***
Pancytopenia	2(8%)	12(48%)	9 (45%)	**χ^2 =10.94**	**0.004***
B-symptoms[a]	2(8%)	15 (60%)	14(70%)	**χ^2 =21.20**	**<0.001***

χ^2 Chi square test, *F* ANOVA test, *U* Mann Whitney test, *yrs* years, *ARD* autoimmune rheumatic disease, *RA* Rheumatoid arthritis, *SLE* Systemic lupus erythematosus, *LPD* lymphoproliferative disorders, *HL* Hodgkin's lymphoma, *B-CLL* B- Chronic lymphocytic leukemia, *NHL* Non-Hodgkin's lymphoma, *DLBCL* Diffuse large B-cell lymphoma, *MCL* Mantle cell lymphoma, *FL* follicular lymphoma, *DAS28* disease activity score 28, *BILAG-2004 index* Index system for assessment of SLE activity, *LN* lymph node, *HSM* hepatosplenomegaly

*Statistically significant at $p \leq 0.05$

[a]B-symptoms includes sweating, weight loss and fever

Relation between cytokine gene polymorphism with risk factors in ARD+LPD patients (group III) (Table 6)

\The relation between TNF-α -308G>A and IL-10 -1082 A>G alleles and genotypes in group III (ARD+LPD) patients with several risk factors is studied Table 5. The factors included gender; time duration of ARD before LPD diagnosis; laboratory data such as ESR, CRP, and RF; and clinical data as autoimmune hemolytic anemia (AIHA). TNF-α -308A allele and -308AA genotype were significantly expressed in ARD female patients who developed LPD more than in males (p = 0.013, p = 0.049, respectively). A significantly higher mean RF titer was detected in

Table 2 Laboratory data of the three studied groups

	Group I (n = 25)	Group II (n = 25)	Group III (n = 20)	Test of Sig.	p
ANA					
1 \64	20 (80%)	22 (88%)	10 (50%)	**χ^2=23.25***	**<0.001***
1 \40	5 (20%)	0 (0%)	1 (5%)		
1 \640	0 (0%)	0 (0%)	2 (10%)		
1 \180	0 (0%)	3 (12%)	7 (35%)		
Anti-DNA					
Median (Min. – Max.)	104 (64-145)	–	64 (64 – 180)	U =30.0	0.408
Mean ± SD.	104.3 ± 35.6	–	99.4 ± 49.2		
RF					
Median (Min. – Max.)	42 (18 – 64)	–	72 (64 – 80)	**t =5.095***	**<0.001***
Mean ± SD.	42 ± 15.5	–	72 ± 8.6		
Anti CCP					
Median (Min. – Max.)	72 (55 – 94)	–	79 (64 – 95)	t =1.356	0.188
Mean ± SD.	72 ± 12.9	–	79.3 ± 11.7		
ESR					
Median (Min. – Max.)	88 (35-118)	74(33 – 130)	96 (75 – 126)	**H =10.051***	**0.007***
Mean ± SD.	$81.8^{b\#}$ ± 27.2	73.4^{b} ± 30.7	$101^{a\#}$ ± 17.3		
CRP					
Median (Min. – Max.)	53 (12 – 95)	34 (14 –114)	72 (32 – 105)	**H =6.010***	**0.049***
Mean ± SD.	$52.6^{b\#}$ ± 24.9	50.8^{b} ± 34.5	$71.7^{a\#}$ ± 23.9		
Ferritin					
Median (Min. – Max.)	–	627.5(430–725)	618 (436 – 724)	t =0.266	0.792
Mean ± SD.	–	591.7 ± 97.5	602 ± 105.4		
LDH					
Median (Min. – Max.)	–	556(290 – 826)	543 (358 – 753)	t =0.116	0.908
Mean ± SD.	–	541.7 ± 171.7	536 ± 131		
β2-M					
Median (Min. – Max.)	–	63.5 (43 – 142)	59 (46 – 73)	U =190.0	0.507
Mean ± SD.	–	75.8 ± 32.4	60.8 ± 8.4		
AIHA	1 (4%)	10 (40%)	6 (30%)	**χ^2=9.31***	**0.01***

χ^2 Chi square test, *F* ANOVA test, *U* Mann Whitney test, *t* student t-test, *H* Kruskal Wallis test, # means with common letters are not significant (i.e. means with different letters are significant), *ARD* autoimmune rheumatic disease, *LPD* lympho-proliferative disease, *yrs* years, *ANA* anti-nuclear antibody, *RF* Rheumatoid factor, *Anti-CCP* anti-cyclic citrullinated protein, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *LDH* lactate dehydrogenase, *β2-M* β2- microglobulin, *AIHA* autoimmune hemolytic anemia

* Statistically significant at $p \leq 0.05$

ARD patients with -308A allele and -308AA genotype (p = 0.027, p = 0.009, respectively). For IL-10 -1082A>G, -1082AA carriers had a significantly longer mean time duration of ARD before LPD diagnosis compared to -1082GG or AG genotypes (p = 0.002). Significantly higher mean ESR (p = 0.049), CRP (p = 0.021), and RF titer (p < 0.001) were detected in ARD patients with LPD who were -1082A compared to -1082G allele carriers. Significantly higher mean RF titer was detected in ARD patients with LPD carrying -1082AA vs 1082GG genotype. No significant relation was detected with any of the studied SNPs and AIHA.

Discussion

The current study studied the proposal that cytokine genetic variation in ARD may contribute to an increased risk of lymphomagenesis development suggesting a common pathological pathway. Our results showed that the frequency of the combined genetic variations, high TNF-α producer (-308A and AA) and low IL-10 producer (-1082A and AA), were significantly higher in ARD patients who developed LPD compared to ARD patients with no malignancy history. This combined cytokine phenotype was associated with severity of ARD (as

Table 3 Distribution frequency of the cytokine genes polymorphisms alleles

Cytokine polymorphism alleles	Group I (*n* = 50) No. (%)	Group II (*n* = 50) No. (%)	Group III (*n* = 40) No. (%)	χ^2	p	OR# (95%CI)
TNF-α promoter						
-308G	27 (54)	11 (22)	12 (30)	**11.95**	**0.003***	**0.37 (0.15-0.88)**
-308A	23 (46)	39 (88)	28 (70)			**2.74 (1.14-6.57)**
	p1=0.022*	p2= 0.001*	p3=0.387			
TGF-β1 codon gene						
Codon 10						
10T	27 (54)	29 (58)	26 (65)	1.12	0.572	1.58 (0.67 – 3.72)
10C	23 (46)	21 (42)	14 (35)			0.63 (0.27 – 1.49)
Codon 25						
25G	45 (90)	43 (86)	36 (90)	0.508	0.776	1.0 (0.25-3.99)
25C	5 (10)	7 (14)	4 (10)			1.0 (0.25–3.99)
IL-10 promoter						
-1082						
A	24 (48)	39 (88)	30 (75)	**11.93**	**0.003***	**3.25 (1.31-8.04)**
G	26 (52)	11 (22)	10 (25)			**0.31 (0.12 – 0.76)**
	p1=0.009*	p2=0.002*	p3=0.74			
-819						
T	11 (22)	21 (42)	17 (42.5)	5.78	0.06	2.6 (1.05 – 6.56)
C	39 (88)	29 (58)	23 (57.5)			0.38 (0.15 – 0.95)
-592						
A	17 (34)	21 (42)	18 (45)	1.25	0.53	1.59 (0.67 – 3.73)
C	33 (66)	29 (58)	22 (55)			0.63 (0.27 – 1.48)
IL-6 Promoter						
-174G	31 (62)	37 (74)	20 (50.0)	5.507	0.064	0.61 (0.26 – 1.42)
-174C	19 (38)	13 (25)	20 (50.0)			1.63 (0.7 – 3.79)
IFN-γ gene:						
+874T	25 (50)	27 (54)	22 (55)	0.264	0.876	1.22 (0.53 – 2.81)
+874A	25 (50)	23 (46)	18 (45)			0.82 (0.35 – 1.88)

χ^2 Chi square test, *TNF-α* tumor necrosis factor- alpha, *TGF-β1* transforming growth factor-β, *IL-10* interleukin-10, *IL-6* interleukin-6, *IFN-γ* interferon-gamma
p: *p* value for comparing between the studied groups. *: Statistically significant at $p \leq 0.05$,
p1: *p* value for comparing between Group I and Group III
p2: *p* value for comparing between Group I and Group II
p3: *p* value for comparing between Group II and Group III
OR#: Odds ratio calculated for Group I and Group III
95%CI= 95% confidence interval

concluded from studying the activity markers). We suggest that this combined phenotype may participate in the supply of a proinflammatory milieu which may increase the risk of lymphomagenesis in ARD patients.

The unregulated chronic B cell antigenic stimulation has been described in autoimmunity-related lymphomagenesis, which summons the clinical importance of studying the underlying genetics driving this pathogenic mechanism [19]. Several genetic polymorphisms have been identified in the TNF-α promoter, a potent proinflammatory cytokine, and affected its production levels [28]. TNF -308G>A was shown to increase gene transcription six- to sevenfold due to modification of the consensus sequence for the transcription factor activator protein (AP-2) binding site, where the common -308G allele has a lower transcriptional activity compared to -308A allele that produces a higher TNF-α serum level [28–30].

The current study showed that ARD+LPD patients had a significantly higher frequency of TNF-α -308A allele and AA+AG genotypes than ARD patients, with 2.7 and 6 times increased risk of LPD development in ARD patients expressing the corresponding alleles and

Table 4 Distribution frequency of cytokine genes polymorphisms alleles among RA/SLE patients in group I and III

	RA				χ^2 (p)	OR	SLE				χ^2 (p)	OR
	Group I (n = 30)		Group III (n = 18)				Group I (n = 20)		Group III (n = 22)			
	No.	%	No.	%			No.	%	No	%		
TNF-α promoter												
-308G	15	50.0	3	16.7	**5.3 (0.02)***	**0.2 (0.05 -0.84)**	12	60.0	9	40.9	1.53 (0.22)	0.5 (0.13-1.58)
-308A	15	50.0	15	83.3		**5.0 (1.19-20.9)**	8	40.0	13	59.1		2.17(0.63-7.44)
TGF-β1 codon gene												
10T	11	36.7	11	61.1	2.69 (0.1)	2.71(0.81-9.05)	16	80.0	15	68.1	0.76 (0.38)	0.54 (0.13 - 2.2)
10C	19	63.3	7	38.9		0.37 (0.11-1.23)	4	20.0	7	31.8		1.87(0.45 – 7.7)
25G	25	83.3	18	100.0	3.35 (0.067)	7.2 (0.403-140.3)	20	100.0	19	86.4	2.94(0.86)	0.16(0.01- 3.4)
25C	5	16.7	0	0.0		0.14 (0.01-2.7)	0	0.0	3	13.6		6.3(0.3-134.6)
IL-10 promoter												
-1082 A	10	36.7	13	72.2	**6.82 (0.009)***	**5.2(1.44-18.7)**	14	70.0	17	77.3	0.3(0.6)	1.5 (0.37-5.8)
-1082 G	20	63.3	5	27.8		**0.2 (0.05-0.7)**	6	30.0	5	22.7		0.7 (0.17-2.7)
-819T	7	23.3	9	50.0	3.6 (0.06)	3.3 (0.93-11.5)	4	20.0	9	40.9	2.14 (0.143)	2.7 (0.69-11.1)
-819C	23	76.7	9	50.0		0.3 (0.09-1.07)	16	80.0	13	59.1		0.36 (0.09-1.44)
-592A	9	30	9	50.0	1.92 (0.17)	2.3 (0.70-7.8)	8	40.0	9	40.9	0.004(0.95)	1.04 (0.3-3.57)
-592C	21	70	9	50.0		0.43 (0.13-1.44)	12	60.0	13	59.1		0.96 (0.28-3.31)
IL-6 promoter												
-174G	19	63.3	11	61.1	0.024(0.88)	0.91 (0.27 -3.03)	12	60.0	9	40.9	1.53 (0.216)	0.46 (0.13-1.58)
-174C	11	36.7	7	38.9		1.1 (0.33 -3.66)	8	40.0	13	59.1		2.2 (0.63-7.44)
IFN-γ gene:												
+874T	17	56.7	9	50.0	0.20 (0.65)	0.77 (0.23-2.5)	8	40.0	13	40.9	1.53 (0.216)	2.2 (0.63-7.44)
+874A	13	43.3	9	50.0		1.31 (0.40-4.22)	12	60.0	9	59.1		0.46 (0.13-1.58)

χ^2 Chi square test, *OR* Odds ratio, *p* p value for comparison between the two studied groups, *RA* Rheumatoid arthritis, *SLE* Systemic lupus erythematosus, *TNF-α* tumor necrosis factor- alpha, *TGF-β1* transforming growth factor-β, *IL-10* interleukin-10, *IL-6* interleukin-6, *IFN-γ* interferon-gamma
*Statistically significant at $p \leq 0.05$

genotypes, respectively. These data suggested that -308A may be a risk factor for LPD development in ARD patients. Meanwhile, ARD patients had a significantly higher frequency of the low-secreting TNF-α -308G allele and GG genotype compared to LPD alone or ARD+LPD, suggesting that TNF -308G allele may be a protective allele in ARD.

Several studies focused on the association between increased TNF inflammatory activity in ARD and increased risk of hematopoietic malignancies [31, 32]. A pooled analysis of several case-control studies presented by Wang et al. concluded that the presence of TNF-α -308 AG or AA genotype in autoimmune conditions increased NHL risk 3.27 times [18]. The crucial role played by TNF-α in B cell development, maturation, and proliferation explains the harmful impact of increased TNF-α circulating levels on the inflammatory immune process, with increased risk of hematopoietic malignancies development [33, 34].

In accordance with our data, Baecklund et al. concluded that increased inflammatory activity was a risk factor for the development of lymphoma in RA patients [35]. The increased inflammatory activity in RA patients was significantly correlated with TNF-α concentrations in serum and synovial fluid of the patients [36, 37]. Yang et al. showed that TNF-α AA and AG genotypes were associated with SLE susceptibility compared with the GG genotype, and the detected increased risk of lupus nephritis was associated with -308A allele [38]. In addition, Vincent et al. study showed that overexpression of the TNF family members, B cell-activating factor (BAFF) and proliferation-inducing ligand (APRIL), correlated with the increased risk of hematologic malignancies in SLE [39]. Hence, TNF-α genetic variation may aggravate ARD inflammatory process, which consequently may increase lymphomagenesis risk.

IL-10 is a potent anti-inflammatory cytokine where the decreased level of IL-10 was associated with severe forms of RA or SLE [40, 41]. Additionally, IL-10 role in tumorigenesis has been demonstrated, where IL-10 deficiency can cause an increase in pro-inflammatory cytokines, hindering the anti-tumor immunity and promoting tumor growth [42]. IL-10 promoter polymorphisms were studied

Table 5 Distribution frequency of TNF -308G>A, IL-10-1082A>G genotypes and IL-10 -1082A>G, -819 T>C and -592 G>C haplotypes in ARD (group I), and ARD+LPD (group III) patients

Cytokine polymorphism genotype	Group I (n = 25)	Group III (n = 20)	χ^2	p	OR (95%CI)
	No. (%)	No. (%)			
TNF-α promoter					
-308 GG	10 (40)	2 (10)	5.13	0.08	0.17 (0.03 - 0.88)
-308AA	8 (32)	10 (50)			2.1 (0.63 - 7.16)
-308GA	7 (28)	8 (40)			1.7 (0.5 – 5.98)
-308GG	10 (40)	2 (10)	5.11	**0.02***	**0.17(0.032-0.88)**
-308 AA+GA	15 (60)	18 (90)			**6.0 (1.13-31.7)**
-308 AA	8 (32)	10 (50)	1.5	0.22	2.13(0.63-7.16)
-308GA+GG	17(68)	10 (50)			0.47 (0.14-1.58)
-308 GA	7 (28)	8 (40)	0.72	0.40	1.71 (0.49-5.98)
-308AA+GG	18 (72)	12 (60)			0.58 (0.17-2.04)
IL-10 promoter					
-1082 AA	7 (28)	12 (60)	5.42	0.07	3.86 (1.11 – 13.46)
-1082 GG	8 (32)	2 (10)			0.24 (0.04 – 1.27)
-1082 GA	10 (40)	6 (30)			0.64 (0.18 – 2.24)
-1082AA	7 (28)	12 (60)	4.66	**0.03***	**3.9 (1.1-13.46)**
-1082GG+GA	18 (72)	8 (40)			**0.26 (0.07-0.90)**
-1082GG	8 (32)	2 (10)	3.114	0.08	0.24 (0.04-1.27)
-1082GA+AA	17 (68)	18 (70)			4.2 (0.78-22.85)
-1082GA	10 (36)	6 (30)	0.48	0.49	0.64 (0.18-2.2)
-1082 AA+GG	15 (64)	14 (70)			1.56 (0.45-5.41)
IL10 promoter Haplotype	(n = 50)	(n = 40)			
ATA	8 (16)	14(35)	4.34	**0.04***	**2.8 (1.04-7.66)**
ACC	16 (32)	10(25)	0.53	0.47	0.71 (0.28-1.80)
GCC	18 (36)	8(16)	2.77	0.09	0.44 (0.17 – 1.17)

χ^2 Chi square test, *TNF-α* tumor necrosis factor- alpha, *TGF-β1* transforming growth factor-β, *IL-10* interleukin-10, *IL-6* interleukin-6, *IFN-γ* interferon-gamma
p: p value for comparing between the studied groups
OR: Odds ratio for Group I and Group III
95%CI= 95% confidence interval
*Statistically significant at $p \leq 0.05$

in relation to IL-10 production and serum levels and showed that IL-10-1082GG and -AG genotypes generated the high IL-10 producer phenotypes, while IL-10- 1082 AA genotypes generated the low producer phenotypes [25, 41, 43, 44].

The current study showed that ARD+LPD patients had a significantly higher frequency of the low-secreting IL-10 -1082A allele and AA genotype with an increased risk of LPD development in ARD patients carrying the corresponding allele and genotype. These data suggested that -1082A allele may be a risk factor for LPD development in ARD patients due to decreased levels of IL-10. Consistently, the increased prevalence of the high-secreting IL-10 -1082G allele in ARD patients compared to other groups suggested that -1082G may be a protective factor in ARD against LPD development due to downregulation of the inflammatory process by increased IL-10 production.

In this study, we focused on the three main studied IL-10 haplotypes: ATA, ACC, and GCC, that gave rise to low-, moderate-, and high-producing phenotypes, respectively [45, 46]. In accordance with our initial observation, our data showed that the low IL-10 producer ATA haplotype was significantly associated with ARD+LPD patients compared to ARD patients with 2.8 times increased risk for LPD development in ARD patients with ATA haplotype.

Several studies showed a significant association between IL-10 -1082 G>A polymorphism and RA or SLE susceptibility [40, 47]; however, the pathogenetic role of IL-10 in lymphomagenesis is controversial. It has been postulated that the lower expression of IL-10 may

Table 6 Relation between TNF-α -308 G>A and IL-10 -1082 A>G alleles/genotype with risk factors in group III

	TNF-α – 308G>A		Test of Sig.(p)	TNF-α – 308G>A			Test of Sig.(p)	IL-10 -1082A>G		Test of Sig.(p)	IL-10 -1082A>G			Test of Sig.(p)
	A(*n* =28)	G(*n* =12)		AA (*n*= 10)	GA (*n* = 8)	GG (*n* = 2)		A (*n*=30)	G (*n*=10)		AA(*n*=12)	GA (*n*=6)	GG (*n*=2)	
Sex														
Male	9 (32.1%)	9(75%)	**χ^2=6.23* (0.013*)**	2 (20%)	5 (62.5%)	2(100%)	**χ^2 =5.96* (0.049*)**	11(36.7%)	7 (70%)	χ^2 =3.37 (0.07)	4 (33.3%)	3 (50%)	2(100%)	χ^2 =3.17 (0.21)
Female	19(67.9%)	3(25%)		8 (80%)	3 (37.5%)	0 (0%)		19(63.3%)	3 (30%)		8 (66.7%)	3 (50%)	0 (0%)	
Years[a]														
median(range)	8(4 – 11)	8(5.5-9)	U =112.0 (0.18)	8(7 -11)	7(5.5-9)	8.5(8-9)	H =3.02 (0.22)	8(4 –11)	6.8(4-8)	U =76.0 (0.17)	8.1(5.5-11)	4.8 (4-5.5)	6.5 (6-7)	**H =12.7* (0.002*)**
mean± SD.	7.4 ± 2.2	8 ± 1.6		7.6 ± 2.4	5.5 ± 1.8	8.5 ± 0.7		7.6 ± 2.04	6.4 ± 2.0		$8.0^{ab¥}$± 1.8	$4.7^{b¥}$ ± 0.9	$6.5^{a¥}$± 0.7	
ESR														
median(range)	96(75-126)	92(88-115)	U=114.0 (0.59)	96(75 –126)	115 (92 –115)	88.3(87.5-89)	H =1.52 (0.47)	106(84-126)	85.5(75-115)	**U =60.0* (0.049*)**	104(84–126)	106(96–115)	75.5(73–78)	H =5.19 (0.075)
mean ±SD.	101.5±18.5	99.6± 13.3		99.8 ± 20.5	107.3 ± 12	88.3 ± 1.1		104.1 ± 16	90.3 ± 17.8		104 ± 17	105.5± 11	75.5± 3.5	
CRP														
median(range)	72(32-105)	72(46-94)	U=122.0 (0.79)	48(44-52)	94(72-94)	66(32-105)	H =5.18 (0.075)	77.5(46-105)	42.5 (32–94)	**U =52.0* (0.02*)**	78(46– 105)	74(53– 94)	33(31-35)	H =5.19 (0.075)
mean± SD.	72.2 ± 24.3	70.4 ± 23		48 ± 5.5	86.7 ± 11.4	67.8 ± 26.3		77.1± 19.8	52.8 ± 27.1		77.7 ± 20	73.5 ± 24	33± 2.8	
RF	(*n* = 15)	(*n* = 3)		(*n* = 6)	(*n* = 3)	(*n* = 0)		(*n* = 13)	(*n* = 5)		(*n* = 6)	(*n* = 0)	(*n* = 3)	
median(range)	112(64-128)	64(64-64)	**t =2.47* (0.027*)**	128(80-128)	64(64-64)	-	**t =3.83* (0.009*)**	128 (80-128)	64(64-64)	**t =5.86 (<0.001)**	128(80-128)	–	64(64-64)	**t =3.83* (0.009*)**
mean± SD.	128 ± 27.7	64 ± 0.0		120± 19.6	64 ± 0.0	-		120±18.7	64±0.0		120±19.6	–	64±0.0	
AIHA	8	4	χ^2=0.28 (0.59)	2	4	-	χ^2=4.80 (0.09)	10	2	χ^2=0.32 (0.57)	4	2	-	χ^2=1.50 (0.47)

χ^2 Chi square test, *U* Mann Whitney test, *H* H for Kruskal Wallis test, *t* student t-test

[#]Time duration of ARD in years before LPD diagnosis. [¥]means with common letters are not significant (i.e. means with different letters are significant). p: *p* value for comparing between the studied groups. *Statistically significant at $p \leq 0.05$

escalate lymphoma risk through being less capable in down tuning TNF-α proinflammatory effect with consequent aggravation of inflammation [48, 49]. Thus, it is comprehensive that these combined genetic factors may act conjointly to escalate chronic inflammation that eventually leads to lymphomagenesis. In accordance with our findings, Wang et al. studies showed that NHL risk increased among autoimmune patients with both variant alleles, TNF-α G308A that produces higher TNF levels, and IL-10 T3575A that produces lower IL-10 levels [18, 50]. Similarly, Cunningham et al. reported that the low-producing IL-10 -1082AA genotype and ATA haplotype were significantly associated with aggressive lymphoma subtypes and contributed to the disease pathogenesis [51]. Another study proposed by Rothman et al. assessed nine immune and inflammatory genetic variants and their association with NHL risk [48]. The study adopted the hypothesis that the lower expression of IL-10 was less capable of suppressing the proinflammatory cytokines; TNF-α and other macrophage proinflammatory cytokines hence increased LPD risk [48]. In support to this hypothesis, several studies depicted the interplay between TNF-α and IL-10 in controlling inflammatory milieu [52, 53]. Degboe et al showed that anti-TNF treatments implemented their effect in improving RA inflammatory process through acting on IL-10 /STAT3 axis by increasing IL-10 production which inhibited TNF-α [53].

Though other studies have suggested that IL-10, as a B cell stimulatory cytokine, may endorse lymphomagenesis, Lan et al. reported that the IL-10 -1082GG genotype was significantly associated with an increased risk for NHL in female Americans [54]. Meanwhile, Berglund et al. [55] and Kube et al. [56] studies concluded that the IL-10-1082A/G SNP was not associated with the increased susceptibility to NHL in German or Swedish populations, respectively. Though, there is no complete agreement on IL-10 role in endorsing lymphomagenesis, the apparently contradictory reviews highlighted that dysregulation of IL-10 cannot be ignored as a risk factor to lymphomagenesis.

We observed that the significant difference in the frequency of both TNF-α -308 G>A and IL-10 -1082 A>G allelic distribution was detected mainly in RA patients, where the frequencies of TNF-α -308A and IL-10 -1082A were significantly higher in RA patients who developed LPD compared to RA patients with no LPD, which will be taken in consideration in our further prospective study.

The present study showed resemblance in the distribution frequency of the studied cytokine gene alleles and genotypes among LPD and ARD+LPD patients. This can propose the susceptibility that both diseases could share the same genetic predisposition; however, this suggestion remains to be elucidated.

Studying the relation between TNF-308G>A and IL-10-1082A>G alleles and genotypes in ARD+LPD with gender demonstrated a significant expression of -308AA carriers in females compared to males. However, this was not in accordance with the recorded increased risk of lymphoma in males [57]. Yet, our recording can be related to the general increased risk of ARD in females and the selected studied sample that showed female predominance. Our data confirmed a significant relation between TNF-308A, -308AA, IL-10-1082A, and -1082AA with ARD activity markers as the increase in RF titer, ESR, and CRP. This was in accordance with the proposal that the increased inflammatory activity was an important determinant in hematological malignancies [35].

We must recognize that a possible limitation of our study was the relatively small sample size of the target studied group (ARD+LPD) which is explained by the low prevalence of this disease category. Nevertheless, we believe that our results remain of considerable interest and should be viewed as exploratory until confirmed in larger studies. Studying of association between cytokine genetic variations and specific lymphoma subtypes is highly recommended, as different histological subtypes have different risk factors. Additionally, it is important to show the translational relevance of the studied SNPs and how the altered cytokine protein levels are associated with lymphomagenesis.

Conclusion

From the present study, it can be concluded that in ARD patients, the significantly higher frequency of the combined cytokine profile of a high TNF-α producer (-308A and AA) and low IL-10 producer (-1082A and AA) genetic variants may participate in the provision of a proinflammatory milieu that may increase the risk of LPD development in this patients group. This combined cytokine phenotype was associated with severity of ARD as reflected on the inflammatory markers. IL-10 and TNF-α are crucial immunoregulatory cytokines which propose them as highly promising genes for the study of lymphomagenesis in ARD. To the best of our knowledge, this study is the first that has investigated the relation of this panel of cytokine gene polymorphisms with an increased risk of LPD development in ARD Egyptian patients.

Abbreviations

AIHA: Autoimmune hemolytic anemia; ARD: Autoimmune rheumatic diseases; CLL: Chronic lymphocytic leukemia; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; HL: Hodgkin lymphoma; IFN-γ: Interferon-gamma; IL-: Interleukin; LPD: Lymphoproliferative disorders; M: Major allele; m: Minor allele; NHL: Non-Hodgkin lymphoma; PCR-SSP: PCR-sequence-specific primer; RA: Rheumatoid arthritis; RF: Rheumatoid factor; SLE: Systemic lupus erythematosus; SNPs: Single nucleotide polymorphisms; TGF-β1: Transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha

Acknowledgements

None.

Authors' contributions
MT conceived of the study and participated in its design. AN supervised the selection of cases and controls. IE, MH, and NZ participated in the design and coordination of the study, supervised the selection of cases and controls, and helped in drafting the manuscript. AF and NS carried out the molecular genetic work, the statistical analysis and interpretation, and drafted the manuscript. All authors read and approved the final manuscript and accepted the publication.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials
The data used or analyzed during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study has been carried out following the approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, in accordance with the Declaration of Helsinki (reference number:020753). Informed written consent to participate in the study was obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 24 September 2019 Accepted: 17 December 2019
Published online: 10 January 2020

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