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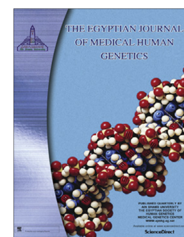

ORIGINAL ARTICLE

Interleukin 10 gene promoter polymorphism and risk of diffuse large B cell lymphoma (DLBCL)

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Abstract
Purpose: Given the importance of understanding the genetic variations involved in the pathogenesis of non-Hodgkin's lymphoma (NHL), this work was designed to study the impact of IL-10 (−1082 G/A; rs1800896 and −819 C/T; rs1800871) gene promoter polymorphism on susceptibility of Egyptians to diffuse large B cell lymphoma (DLBCL); the major type of NHL. To the best of our knowledge, this study is the first one that examines IL-10 promoter polymorphism in DLBCL in Egyptians.

Methods: Genotyping polymorphism is performed using sequence-specific primers polymerase chain reaction (SSP-PCR) in 100 Egyptian DLBCL patients and 119 normal controls. Circulating plasma levels of IL-10 were measured using Enzyme-linked immunosorbent assay (ELISA).

Results: Insignificant change in IL-10 (−1082 and −819) genotypes was recorded. Although A allele is slightly decreased in DLBCL patients, it did not reach statistical significance. GT haplotype was significantly elevated ($P < 0.05$) in NHL patients. A significant linkage disequilibrium between the −1082 and −819 SNPs with $D' = 0.596$ and $r^2 = 0.1032$ ($P < 0.001$) was demonstrated. Significantly increased plasma IL-10 ($P < 0.01$) was found which is positively correlated ($r = 0.307$; $P < 0.01$) with the disease.

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Conclusions: Taken together, our findings demonstrated that IL-10 promoter gene polymorphism (−1082 and −819) may not have an influence on the clinical outcome of DLBCL, especially in terms of overall secretion level. Further investigations of other cytokine gene polymorphisms will lead to a better understanding of the disease's biological background.

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1. Introduction

Non-Hodgkin's lymphoma is a heterogeneous group of hematological malignancies [1,2]. It represents the sixth most common cause of cancer death worldwide [2]. Approximately 287,000 new cases of NHL are reported in the world each year [3]. Several subtypes of NHL were characterized. The two major subtypes are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) [4]. Diffuse large B cell lymphoma (DLBCL) accounts for approximately one third of all adult NHL cases [5]. The interaction of the host immune response with NHL and the impact of this interaction on the clinical outcome of the disease are not yet fully understood.

Many cytokines are known to be involved in the pathogenesis of NHLs [6]. Recent studies suggested that several cytokine gene polymorphisms were associated with susceptibility to NHL [7] including interleukin-10 which acts as a growth factor for normal activated human B and T lymphocyte stimulation and proliferation [4,8,9]. IL-10 has been found to act as an autocrine growth factor which up-regulates apoptosis regulator Bcl-2 expression in some B-cell malignancies [10,11]. Some evidences suggest that IL-10 might be associated with the progression of T-cell NHLs and in the pathogenesis of DLBCL as IL-10 may be involved in a rescue effect, protecting T cells from apoptotic cell death associated with up regulated bcl-2 expression [12].

The gene encoding IL-10 is located on chromosome 1 (1q31–1q32) [13,14]. It has been reported that 3 SNPs in the IL-10 gene promoter, including (−1082, −819, and −592) may influence IL-10 production in vitro [15]. Studies of Skibola et al. [4] and Kube et al. [16] suggested that IL-10 gene polymorphisms were associated with susceptibility to NHL. Recently, a meta-analysis of Cao et al. [17] suggested that IL-10 −3575 A allele confers a greater risk to DLBCL susceptibility, while −1082 A/G polymorphism may have a significant association with DLBCL risk. However, no such study was performed on Egyptian population. Thus, this study was conducted to investigate the genotype distribution of IL-10 (−1082 G/A; rs1800896 and −819 C/T; rs1800871) in DLBCL Egyptian patients in view of its potential contribution to the pathogenesis of the disease. Plasma level of IL-10 was measured in the same patients and control groups to determine whether the change in the level of these cytokines is associated or independent of the genetic markers at these polymorphic sites.

2. Subjects and methods

2.1. Patients and controls

This study was conducted on 100 DLBCL patients (58 men and 42 women; mean age 50.32 ± 14.58 years; range 17–79 years) recruited consecutively from the Oncology Hospital, Menufia University (Shebein El-kom, Menufia Governorate, Egypt). One hundred nineteen unrelated healthy blood donors free of any chronic diseases, living in the same geographical area and having the same ethnic origin as patients were recruited as normal healthy controls. Informed consent was obtained from all the study subjects. All investigations were done in accordance with the Menufia University, Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethics committee approved the study protocol.

Cases were categorized according to the WHO classification [1] and the diagnosis of NHL was confirmed by histology and immunohistochemistry tests. The initial medical evaluation consisted of a complete history and physical examination; chest radiographic examination; computed tomographic scan of the chest, abdomen, and pelvis; blood chemistry and bone marrow biopsy or aspirate was performed at diagnosis. The extent of the disease was categorized according to the Ann Arbor classification and performance status was assessed using criteria of the Eastern Cooperative Oncology Group (ECOG) [18].

2.2. DNA isolation

Blood samples were collected on ethylene-diamine-tetra-acetic acid (EDTA) tube by venipuncture from all subjects. Genomic DNA was extracted from whole blood-EDTA samples using Wizard® Genomic DNA Purification Kit (Promega Co., WI, USA) according to the manufacturer's instructions.

2.3. Genotyping

IL-10 (−1082 G/A and −819 C/T) SNPs were analyzed by sequence-specific primers polymerase chain reaction (SSP-PCR) as an ARMS-PCR (amplification mutation (refractory system.) using four primer mixtures [19]. For −1082 G/A, primer G (sense): 5''-CTACTAAGGCTTCTTTGGGAG-3′ primer; A (sense): 5''-ACTACTAAGGCTTCTTTGGGAA-3′ and antisense: primer (antisense): 5''-CAGTGCCAACTGAGAATTTGG-3′ were used. For −819 C/T, primer C (sense): 5′-CCCTTGTACAGGTGATGTAAC-3′; primer T (sense): 5′-ACCCTTGTACAGGTGATGTAAT-3′ and antisense: primer (antisense): 5′-AGGATGTGTTCCAGGCTCCT-3′ were used. As internal control the following primers sense: 5′-GCCTTCCCAACCATTCECTTA-3'' and antisense: 5′-TCACGGATTTCTGTTGTGTTTC-3′ were used. The reaction was done in two tubes one for each allele, the final volume for each PCR reaction was 25 μl. The PCR mixtures consisted of DreamTaq Green PCR Master Mix (2×) (Fermentas, Thermo Fisher Scientific Inc.), 10 pmol of each allele-specific primer, 10 pmol of antisense primer, 3.5 pmol of each control primer and 100 ng of DNA. PCR cycling conditions consisted of 94 °C for 2 min [1 cycle], followed by 96 °C for 25 s, 70 °C for 45 s, and 72 °C for 20 s [5 cycles]; followed by 96 °C for 25 s, 65 °C for 50 s, and 72 °C for 45 s

[11cycles]; and finally 96 °C for 25 s, 55 °C for 60 s, and 72 °C for 2 min [15 cycles]. The control primer resulted in amplicon of 429 bp and the −1082 primers resulted in an amplicon of 258 bp while the −819 primers resulted in an amplicon of 233 bp. By 2% agarose gel, the size of PCR products was determined relatively to the migration of a 100 bp step ladder (Fermentas) (Figs. 1a and 1b).

2.4. Measurement of plasma IL-10 by enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from all patients and controls, plasma separated by centrifugation at 1500 rpm for 15 min at 4 °C, aliquotted, and stored at −80 °C until cytokine analysis. Total concentrations of IL-10 in blood samples were measured using a commercial ELISA kit (R&D System, Inc., Minneapolis, MN), according to the manufacturer's instructions. The intensity of the developed color was measured by reading optical absorbance at 450 nm using a microplate reader (SunriseTM, Tecan Group Ltd. Männedorf, Switzerland)). The ELISA reader-controlling software (Softmax) processed the digital data of raw absorbance values into a standard curve, from which the IL-10 concentration of the samples was derived. Results were expressed as pictogram of cytokine per milliliter plasma (pg/ml).

2.5. Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 13 (LEAD Technology Inc.). Analysis of SNPs was done according to the factors of the international prognostic index [IPI; age [IPI] > 60 years, lactate dehydrogenase (LDH) > N, Eastern Cooperative Oncology Group (ECOG) > 1, stage III/IV > 1 extranodal involvement to be representative for the disease population]. Data were presented as means with corresponding standard deviation (SD). Comparisons among different groups were performed by independent *T*-test. Chi-square test was used to compare the frequency of variables in different groups, and OR and 95% CI were calculated as determined by the program software. Correlation between variables was determined using Sperman's correlation test. *P* value that remained below 0.05 after correction for the number of variables (*P* corrected, *Pc*) was considered significant (Bonferroni correction). The

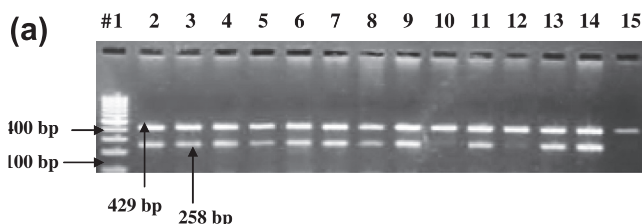

Figure 1a IL-10 (−1082 G/A) PCR products of seven samples. Lane (1) 100 bp ladder, sample 1 in lane (2 and 3), sample 2 in lane (4 and 5), sample 3 in lane (6 and 7) and sample 4 in lane (8 and 9) are GA genotype, sample 5 in lane (10 and 11) and sample 6 in lane (12 and 13) are AA genotype and sample 7 in lane (14 and 15) GG genotype.

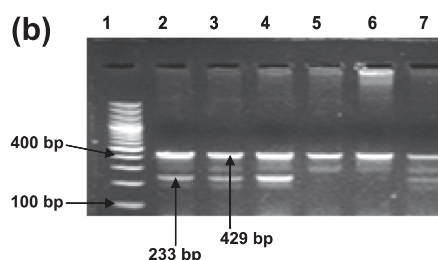

Figure 1b IL-10 (−819 C/T) PCR products of three samples. Lane (1) 100 bp ladder, Sample 1 in lane (2 and 3) CT genotype, sample 2 in lane (4and 5) CC genotype and sample 3 in lane (6 and 7) TT genotype.

haplotype frequencies were estimated with linkage disequilibrium coefficient, *D*, using SNPSTAT program (available at http://bioinfo.iconcologia.net/snpstats /start.htm). *D* was expressed as D' giving the value of *D* as a percentage of the maximum calculated value given the observed allele frequencies. Values of D' ranged between −1 and +1. A/D'/value of 1 denoted complete linkage disequilibrium whereas a value of 0 denoted complete linkage equilibrium.

3. Results

3.1. Patient's characteristics

The detailed biochemical characteristics of the patients enrolled in this study are presented in Table 1.

3.2. Association between IL-10 polymorphisms and NHL

Two pictures of gel electrophoresis of different genotypes of IL-10 are shown in Figs. 1a and 1b. The IL-10 (−1082 G/A and −819 C/T) genotypes and allele frequencies in patients and healthy controls are shown in Table 2. Analysis of SNPs revealed that, there was an insignificant change in the distribution of IL-10 (−1082 G/A and −819 C/T) genotypes between patients and healthy controls. Although A allele is slightly decreased in DLBCL patients, it did not reach statistical significance.

The frequency of IL-10 (−1082/−819) haplotypes in DLBCL patients and healthy controls is shown in Table 3. The AC was the most frequent haplotype in controls and the GC in patients. The GT is the lower one in both groups. There was a significant increase in GT haplotype in DLBCL patients ($P < 0.05$) compared to controls. The linkage disequilibrium (LD) pattern between the −1082 G/A and −819 C/T SNP showed a significant ($P < 0.001$) LD, with a D' value of 0.596 and an r^2 (the coefficient of determination) value of 0.1032.

3.3. Plasma levels of IL-10 in NHL patients

A significant increase ($P < 0.01$) in IL-10 in DLBCL patients compared to healthy controls (56411.08 ± 11802.60 versus 5053.32 ± 279.64) was demonstrated. The disease was significantly correlated with elevation of IL-10 ($r = 0.307$; $P < 0.01$) secretion level.

Table 1 Biochemical characteristics of control and diffuse large B-cell lymphoma (DLBCL) patients.

Laboratory investigations	Control group (N = 119) (Mean ± SD)	DLBCL group (N = 100) (Mean ± SD)	p
WBCs (1000/mm^3)	6.58 ± 2.03	8.53 ± 6.29	$p < 0.01^*$
Hemoglobin (mmol/L)	8.32 ± 1.0	6.55 ± 1.31	$p < 0.001^{**}$
PLT (1000/mm^3)	267.59 ± 63.04	285.72 ± 166.21	NS
AST (IU/L)	21.96 ± 5.85	49.61 ± 43.51	$p < 0.001^{**}$
ALT (IU/L)	17.91 ± 5.05	45.81 ± 50.09	$p < 0.001^{**}$
ALB (μmol/L)	623.82 ± 57.09	527.19 ± 80.12	$p < 0.001^{**}$
Bilirubin (μmol/L)	11.80 ± 3.57	21.92 ± 42.88	$p < 0.01^*$
Creatinine (μmol/L)	76.71 ± 13.69	118.65 ± 107.76	$p < 0.01^*$
LDH (IU/L)	326.61 ± 43.27	548.78 ± 477.67	$p < 0.001^{**}$

All data are presented as mean ± SD. Platelet (PLT), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Albumin (ALB), Lactate dehydrogenase (LDH).
Correlation coefficients in bold are statistically significant with $p < 0.05$.
NS: not significant.
* $p < 0.01$.
** p $p < 0.001$.

Table 2 Genotype distribution and allelic frequency of the IL-10 (−1082 G/A and −819 T/C) in controls and patients with Diffuse large B-cell Lymphoma (DLBCL).

Cytokine gene	Control group N = 119)	DLBCL group (N = 100)	p/pc	OR (95% CI)
IL-10 (−1082 G/A) Genotype (N, %)				
G/G	15 (12.6%)	18 (18.0%)	NS	1.522 (0.723–3.202)
G/A	61 (51.3%)	54 (54.0%)	NS	1.116 (0.655–1.902)
A/A	43 (36.1%)	28 (28.0%)	NS	0.687 (0.387–1.221)
GAAA	104 (87.4%)	82 (82.0%)	NS	0.657 (0.312–1.382)
Allele frequency				
G	91 (38.0%)	90 (45.0%)	NS	1.321 (0.902–1.933)
A	147 (62.0%)	110 (55.0%)	NS	0.756 (0.516–1.108)
IL-10 (−819 C/T) Genotype (N, %)				
C/C	62 (52.1%)	40 (40.0%)	NS	0.613 (0.358–1.050)
C/T	52 (43.7%)	54 (54.0%)	NS	1.513 (0.886–2.582)
T/T	5 (4.2%)	6 (6.0%)	NS	1.455 (0.431–4.919)
CTTT	57 (47.9%)	60 (60.0%)	NS	1.632 (0.953–2.794)
Allele frequency				
C	176 (74.0%)	134 (67.0%)	NS	0.715 (0.473–1.081)
T	62 (26.0%)	66 (33.0%)	NS	1.398 (0.925–2.113)

P: *p*-Value (significant), *Pc*: *P* corrected, *p*/*pc*: gives Bonferroni correction, NS: not significant.

Table 3 Haplotype frequencies of the IL-10 (−1082 and −819) polymorphisms in control and Diffuse large B-cell lymphoma (DLBCL) patients.

Haplotype	Control group (N = 119)	DLBCL group (N = 100) %	p	OR (95% CI)
GC	36.68	36.54	NS	1.00
AC	37.27	30.46	NS	0.82 (0.50–1.35)
AT	24.50	24.54	NS	1.03 (0.59–1.80)
GT	1.55	8.46	**$p < 0.05^*$**	6.46 (1.26–33.13)

$p < 0.01$.
p $p < 0.001$.
P: *p*-Value (significant), *Pc*: *P* corrected, *p*/*pc*: gives Bonferroni correction, NS: not significant.
* Correlation coefficients in bold are statistically significant with $p < 0.05$.

3.4. Differential expression of IL-10 in patients and controls according to polymorphisms

As shown in Table 4, the mean plasma concentration of IL-10 (−1082 G/A) was increased significantly ($P < 0.01/Pc < 0.01$, $P < 0.01/Pc < 0.01$ and $P < 0.001/Pc < 0.001$) in DLBCL patients with GG, GA and AA genotypes; respectively. The same results were observed in IL-10 (−819 C/T) where IL-10 was elevated in patients with CC and CT ($P < 0.01/Pc < 0.01$ and $P < 0.001/Pc < 0.001$, respectively).

4. Discussion

Polymorphisms in genes coding for molecules critical in the disease development and progression are likely to represent key factors in the amplification of intrinsic biological differences, resulting in clinically distinct outcomes [20]. IL-10 is a multifunctional cytokine; it can stimulate proliferation and differentiation of tumor B cells [21] and play an important role in the malignant lymphoma development process [9]. It has been reported that IL-10 SNPs may influence immune function through modulating the activities of NK, T cells and macrophages and thus alter the disease progression [22]. Our results showed that allelic frequencies and genotypic distributions at the IL-10 (−819) polymorphisms did not differ between DLBCL patients and the control group. These data are consistent with those previously reported by Lech-Maranda et al. [18] and Purdue et al. [23]. The same result was found in-L-10 (−1082) which is consistent with those presented by Ghielmini and Mora [24] where the frequency of the IL-10 (−1082) G allele was not significantly different in DLBCL patients versus the control group. In contrast, a contradictory result was obtained by Purdue et al. [23]. Carriers of the IL-10 (−1082) G allele are increased in NHL patients compared with healthy controls and this was in agreement with Lech-Maranda et al. [18] in DLBCL. A recent study suggested that −1082 G/A was no longer statistically significant among Hispanic whites [25]. It is obvious that the investigation on the relationship between IL-10 SNPs and DLBCL was not always consistent. Some studies have found correlations between −1082 A/G and the risk of DLBCL [7,23,26]. However, other groups have failed to confirm these associations [27,28]. This stressed the fact that different ethnic groups have different panels of polymorphisms in certain gene.

In our study, serum IL-10 concentration was significantly higher in patients compared to the healthy controls. Our results were consistent with previously documented data [9,29–31]. Increased serum levels of IL-10 reflect an enhanced activation of the immune system on more aggressive disease, but its potential action as a growth factor for lymphoma cells or as a suppressor of macrophages or T-cell functions should also be taken into account [32]. The increase of IL-10 production within tumor microenvironment might be protective and conversely, that low IL-10 producing capability makes individuals susceptible to more aggressive course of the disease [33,34]. Increased serum levels of IL-10 were found in DLBCL patients and were correlated with adverse disease features and poor DLBCL outcome [35,36]. Although IL-10 gene promoter polymorphisms might affect the levels of IL-10 expression [15], in our work, the increase in IL-10 production levels in lymphoma patients is independent on the genotype of the patient either in IL-10 (−1082) or IL-10 (−819) loci. Our result was consistent with the result obtained from Lech-Maranda et al. [18]. Few studies have linked polymorphism with secretion level, with a lot of inconsistence data. This arise the importance of evaluating the real effect of IL-10 promotor gene polymorphism on IL-10 secretion level in different ethnic origins of several populations.

5. Conclusions

In conclusion, this preliminary data indicated that IL-10 (−1082 and −819) genotypes did not play a role in DLBCL susceptibility in Egyptian patients. The variation documented in this work from other studies in different populations could be returned to the ethnic differences. Therefore, larger prospective studies are needed to confirm our findings. In the light of these data, further studies concerning other cytokine gene polymorphisms will contribute to a better understanding of the pathogenesis of the disease.

Table 4 Mean serum concentrations of interleukin IL-10 according to gene polymorphism (−1082 G/A, −819 C/T) in controls and Diffuse large B-cell lymphoma (DLBCL) patients.

Genotype	Control group (N = 119) (Mean ± SE)	DLBCL group (N = 100) (Mean ± SE)	*p/pc*
IL-10 (−1082 G/A) (pg/ml) (N)			
G/G (15, 18)	5593.6 ± 1193.8	50732.7 ± 11967.3	$\mathbf{p < 0.01/pc < 0.01}$*
G/A (61, 54)	4966.3 ± 340.0	74433.6 ± 21042.5	$\mathbf{p < 0.01/pc < 0.01}$*
A/A (43, 28)	5021.3 ± 498.2	25303.5 ± 5489.5	$\mathbf{p < 0.001/pc < 0.001}$**
IL-10 (−819 C/T) (pg/ml) (N)			
C/C (62, 40)	5087.2 ± 413.7	57227.7 ± 21035.6	$\mathbf{p < 0.01/pc < 0.01}$*
C/T (52, 54)	5187.0 ± 397.2	61648.1 ± 15307.7	$\mathbf{p < 0.001/pc < 0.001}$**
T/T (5, 6)	3242.0 ± 862.7	3833.3 ± 1275.8	NS

Correlation coefficients in bold are statistically significant with $p < 0.05$.
* $p < 0.01$.
** p $p < 0.001$.

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