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BAFF Promoter Polymorphisms and Serum levels in Tunisian Patients with Systemic Lupus Erythematosus

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Abstract

Objectives: To investigate any associations between regulatory genetic polymorphisms of the B-lymphocyte activating factor (BAFF) gene, disease susceptibility and serum soluble BAFF (s-BAFF) levels in Tunisian systemic lupus (SLE) patients.

Patients and methods: This case-control study included 124 SLE patients and 152 healthy controls. Three single nucleotide polymorphisms (SNPs) (-2841 T>C, -2701 A>T and -871 C>T) in the 5' regulatory region of the BAFF gene were explored by PCR-RFLP. Serum BAFF levels were measured by ELISA (R&D Systems®).

Results: s-BAFF levels were elevated in SLE patients (1717.08 pg/ml) and in anti-dsDNA positive antibodies patients (1948.28 pg/ml) compared to both controls (665.82 pg/ml, $p<10^{-8}$) and patients without anti-dsDNA antibodies (1281.51 pg/ml, $p=0.007$). In contrast, no correlation was found between global disease activity registered in SLEDAI and s-BAFF levels ($p=0.7$).

The -2841*C mutated allele was associated to SLE predisposition and anti-dsDNA antibody occurrence, $p=0.03$ and $p=0.021$ respectively. Single allele, genotype and haplotype association analyses showed no significant association with s-BAFF values, clinical features or SLEDAI score.

Conclusion: In Tunisian, the rs9514827 (T>C -2841) SNP in the BAFF gene promoter seems to be related to SLE susceptibility and anti-dsDNA antibodies occurrence. Even if serum s-BAFF levels were significantly higher in SLE patients and in anti-dsDNA antibody positive sera, it did not seem to be correlated with disease activity or the occurrence of lupus nephritis. Again, studied genetic markers failed to predict the serum s-BAFF levels as there was no correlation between the two parameters.

Keywords: BAFF; Polymorphism; Promoter; Serum; Systemic lupus erythematosus; Genetics; SLE disease activity index; Anti-double-stranded DNA

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder in which loss of tolerance to nucleic antigens especially dsDNA is associated with the development of pathogenic antibodies that damage target organs. This aberrant synthesis of auto-antibodies is due to a hyper-activation of self-reactive B-cells. B-cells originate from bone marrow, but must undergo further maturation in periphery. In fact, those B-cells pass through transitional, immature stages in the spleen: first, a transitional type 1 (T1) stage ($IgM^{high}IgD^{-}$), followed by the T2 stage (IgD^{high}) [1]. Only a small proportion of B-cells reach maturity, indicating a vigorous selection process. Signaling through the B-cell receptor (BCR) is important in this selection process, but a lonely BCR signals cell death [2], suggesting the involvement of additional factors such as B-cell activating factor (BAFF). BAFF is a 285-amino-acid glycoprotein originally expressed as a type II membrane-bound protein, which can be released as a soluble trimeric ligand (s-BAFF) upon proteolytic processing at a furin consensus site [3].

The sources of BAFF are primarily innate immune cells such as monocytes, dendritic cells, neutrophils, and stromal cells, including central nervous system (CNS) astrocytes and airway epithelia [4]. These innate immune cells, peculiarly neutrophils, were generally found in most of damaged tissues in SLE [5], but their exact contribution to disease pathogenesis and the mechanisms that affect their amassing are poorly understood. In SLE-mice spleens, neutrophils localized in T-cell zones enhanced $CD4^{+}$ T-cell proliferation and IFN-γ production through BAFF release [5]. In addition, neutrophils depletion led to reduced BAFF and IFN-γ serum levels, extinction of B-cell germinal centers and decreased autoantibody production [5]. Thus, it suggests that targeting neutrophils could be a one of the therapeutic strategies in SLE treatment.

Recent data suggest that BAFF signaling facilitates T2 survival and all above its over-expression resulted in hugely increased numbers of mature, but not immature B-cells [6]. Furthermore, BAFF transgenic mice develop autoimmunity resembling SLE, including hyperplasia of mature B-cells, autoantibodies production and deposition in kidney

[7]. In fact, BAFF over-expression rescues self-reactive B-cells from peripheral deletion allowing maturation and colonization in the follicular and marginal zone, which then enhances autoantibody production [8]. In mice with SLE-like syndrome selective blockade of BAFF, which resulted in B-cells depletion and splenic collapse, was sufficient to prevent the lupus nephritis occurrence [9].

Besides, BAFF co-stimulation drives T-Cell proliferation and up-regulates Bcl-2, an anti-apoptotic factor, in activated T-cells [7]. Over-expression of BAFF promotes the generation of the pro-inflammatory Th17 subset in vivo and in vitro and aggravates the experimental autoimmune encephalitis in mice [10]. Likewise, Intra-articular injection of lentivirus expressing shRNA for BAFF gene silencing provided long-term suppression of autoimmune arthritis development, suppressed Th17 cells generation, and significantly improved joint pathology [11].

Recently and interestingly, many cytokines such as BAFF have been shown to be antibody targets in SLE [4]. Surprisingly, autoantibodies to BAFF were correlated to a more severe SLE profile [12]. This unique result needs to be replicated in larger and independent SLE cohorts. If this association between disease severity and the presence of anti-BAFF antibodies is validated, then the question would be: how a neutralizing autoantibody against a pathogenic cytokine worsens SLE outcome? The reasons would be: first, the BAFF immune complexes induces a positive feedback loop that further increases the cytokine expression; second, the anti-BAFF antibody preferentially binds to dominant-negative form of BAFF, called Δ-BAFF, then the pathogenic effect of this cytokine could be unleashed; third, like any other immune complex, BAFF/anti-BAFF complex may activate the classical pathway of the complement system.

Single nucleotide polymorphisms (SNPs) in the 5' regulatory region the BAFF gene have been identified in patients with SLE [13]. The rs9514828 (C>T -871) SNP was associated to a higher expression of BAFF mRNA in SLE [13] and elevated s-BAFF in Sjögren's syndrome (SS) [14]. The rs9514828 SNP was also found to be in a strong linkage disequilibrium with other promoter SNPs such as the rs9514827 (T>C -2841) and the rs1041569 (T/A -2701) [14,15]. On the other hand, even lacking correlation with SLE activity, BAFF levels were significantly increased in SLE-patients with either renal or CNS involvement [16]. Hence, we performed a case-control study to assess the impact of these 3 SNPs on s-BAFF level and both SLE-susceptibility and SLE-related features in a Tunisian cohort.

Materials and Methods

Patients and controls

This study included 124 SLE patients and 152 healthy voluntary blood donors from the same geographic origin. Patients were visiting both internal medicine departments A and B of the Charles Nicolle Hospital in Tunis and were diagnosed according to the Criteria Committee of the American College of Rheumatology [17]. Clinical and biological features of patients are recorded in Table 1.

SLE patients	n=124
Mean onset age ± SD (years)	31.93 ± 13.86
Median of evolution ± SD (years)	5 ± 4.96
Sex ratio (male/female)	0.17 (18/106)
Cutaneous lesions n (%)	103 (83%)
Arthritis n (%)	102 (82.2%)
Nephritis n (%)	75 (60.4%)
Neurolupus n (%)	15 (12%)
Autoimmune Cytopenias n (%)	83 (66.9%)
Thrombosis n (%)	5 (4%)
Elevated titer of ANA>1/320 n (%)	93 (75%)
Anti-dsDNA antibodies n (%)	81 (65.3%)
Anti-Ribosome antibodies n (%)	16 (12.9%)
Anti-SS-A/Ro-52 antibodies n (%)	47 (37.9%)
Anti-SS-A/Ro-60 antibodies n (%)	37 (29.8%)
Anti-SS-B/La antibodies n (%)	41 (33.1%)
Decreased complement activity (DCA) n (%)	105 (84.7%)
SLEDAI* mean ± SD	11.73 ± 6.05
SLEDAI ≥ 8 n (%)	94 (75.8%)
*SLE disease activity index	

Table1: Clinical and biological features of SLE patients.

Controls were healthy subjects matched for age, gender and ethnicity. None of the healthy subjects had any evidence of personal or family history of autoimmune disease.

All patients and controls gave written informed consent to participate in the study, and patient anonymity was preserved using documents and methods approved by the local Ethics committee of Charles Nicolle Hospital.

Methods

Serum s-BAFF measurement: In both SLE patients and controls sera the s-BAFF was measured by a quantitative sandwich ELISA according to the recommended protocol by the manufacturer (Quantikine® Human BAFF Immunoassay, R&D Systems, Minneapolis, MN, USA). Levels of s-BAFF are expressed in pg/ml. The threshold of elevated s-BAFF was estimated by calculating a cut-off value:

Mean s-BAFF (in controls: 665.82 ± 171.472 pg/ml) + 2 SD=1008.764 pg/ml.

BAFF genotyping: Genomic DNA was extracted from peripheral blood using salting-out procedure [18]. Identification of BAFF (T>C -2841), (T>A -2701) and (C>T -871) SNPs was performed by PCR using specific primers [(-2841*F: 5'-ATTCCCTGTCTTCAGAATTTTCTCT-3' and -2841*R: 5'-CCTATAACTCCCACAATAAGGTGAC-3'), (-2701*F: 5'-ATTCCCTGTCTTCAGAATTTTCTCT-3' and -2701*R: 5'-CCTATAACTCCCACAATAAGGTGAC-3') and (-871*F: 5'-TTGTACACCGACCTGTTAGGC-3' and -871*R: 5'-TGGAAGTAAGTCCACTGGGAAT-3') (metabion® international AG, Lena-Christ-strasse 44I, D-82152 Martinsried, Deutshland)], followed

by a digestion of amplification products using *SsiI* (*AciI*) for (T>C -2841) and (C>T -871) SNPs and *DpnII* For (T>A -2701) SNP (*Restriction fragment length polymorphism:* RFLP). Direct sequencing was made for 10 DNA samples in order to validate the genotyping by PCR-RFLP and reference samples of each genotype were included in each assay.

Statistical analysis

The results of continuous variables (Age, Onset Age and s-BAFF level) are expressed as means ± SD, and the means of groups were compared by ANOVA-test (SPSS 11 Inc. Chicago, Illinois, USA). For qualitative variables, univariate analysis was performed using chi-square test or fisher's exact test for small numbers (Epi-info Stat 6.04 program CDC, Atlanta). Probability (p) values were corrected for the number of tested alleles (pc). Values<0.05 were considered to be statistically significant. Frequencies of genotypes and alleles were analyzed by chi-square test. In order to evaluate the strength of associations, the odds ratios (OR) together with 95% confidence intervals (CI) were calculated. Logistic regression models were built according to age, gender to estimate adjusted ORs. The Hardy-Weinberg equilibrium was verified for the three studied SNPs via internet (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl).

BAFF haplotypes, defined by the 3 SNPs, were analyzed using the freely available SNPStats software (http://bioinfo.iconcologia.net/snpstats/start.htm).

Results

Determination of serum s-BAFF levels

Mean s-BAFF concentrations were significantly higher in SLE patients (1717.08 ± 1322.716 pg/ml) comparatively to controls (665.82 ± 171.472 pg/ml); F=94.223, $p<10^{-18}$ (Figure 1). Using the *cut-off value* (1008.764 pg/ml), 78 (62.9%) patients had an elevated level of serum s-BAFF while in only 3 (1.9%) controls it exceeded the threshold; $p<10^{-27}$, OR (95% CI) = 84.22 [24.09-351.4].

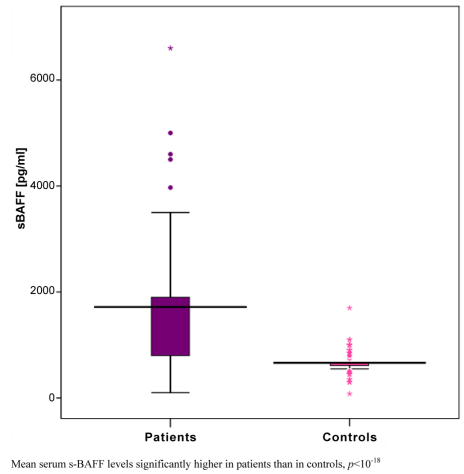

Figure 1: Serum s-BAFF in SLE patients and controls.

Analysis of s-BAFF according to clinical and biological features showed: first, the mean s-BAFF level was significantly higher in men (2561.33 pg/ml) than in women (1573.33 pg/ml), p=0.003, while in controls there was no correlation with gender; second, the presence of anti-dsDNA antibodies was associated to a higher s-BAFF concentration (1948.28 pg/ml vs 1281.51 pg/ml), p=0.007 (Figure 2). Multivariate analysis by logistic regression confirmed the univariate results. Inversely, there were no correlations between s-BAFF levels and SLEDAI, any of SLE clinical manifestations (cutaneous lesions, arthritis, lupus nephritis and neurolupus), elevated titer of anti-nuclear antibodies (ANA) and decreased complement activity (DCA). Moreover, SS-related autoantibodies (Ro/La) occurrence was not correlated to elevated serum s-BAFF.

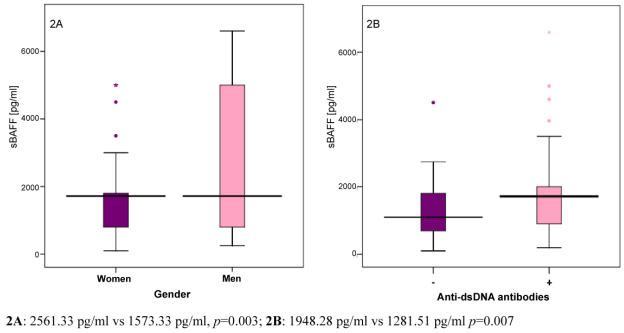

Figure 2: Correlations between s-BAFF and both gender and positivity of anti-dsDNA antibodies.

BAFF SNPs results

If genotypic and allelic frequencies for (T>A -2701) and (C>T -871) SNPs were similar between patients and controls, the -2841*C allele was significantly more prevalent in SLE patients (0.379) than in controls (0.293); p=0.013, OR (95% CI) = 1.57 [1.08-2.29] (Table 2).

BAFF T>C -2841		**Controls n=152**	**SLE n=124**	*p*	**OR (95% CI)**
Genotypes	T/T	78 (50%)	44 (35.5%)	0.053	_
	T/C	63 (41.4%)	66 (53.2%)		
	C/C	13 (8.6%)	14 (11.3%)		
Alleles	T	0.707	0.621	0.013	1.57 [1.08-2.29]
	C	0.293	0.379		
BAFF T>A-2701					
Genotypes	T/T	3 (2%)	5 (4%)	0.581	_
	T/A	48 (31.6%)	37 (29.9%)		
	A/A	101 (66.4%)	82 (66.1%)		
Alleles	T	0.178	0.189	0.431	_
	A	0.822	0.811		
BAFF C>T -871					
Genotypes	C/C	3 (2%)	3 (2.4%)	0.86	_
	C/T	131 (86.2%)	104 (83.9%)		

	T/T	18 (11.8%)	17 (13.7%)		
Alleles	C	0.451	0.444	0.922	–
	T	0.549	0.556		

Table 2: Genotypic and allelic frequencies of the 3 SNPs in SLE patients and controls.

Moreover, the -2841*C mutated allele was consistently associated with an earlier onset of SLE (25.14 vs 30.67, p=0.038), and with the presence of anti-dsDNA antibodies (73.75% vs 50%, p=0.021). Inversely, the 2 other SNPs (T>A -2701) and (C>T -871) exhibited no correlations with clinical and biological features of SLE. None of the 3 SNPs had any influence on the disease activity. In the same way, there was no evidence of any associations of the 3 BAFF SNPs with the s-BAFF levels in either SLE patients or controls; (T>C -2841, p=0.168), (T>A -2701, p=0.515) and (C>T -871, p=0.349) (Figure 3).

The three BAFF SNPs were in strong linkage disequilibrium, p=0.007, and shaped eight different combined haplotypes and among them 4 common haplotypes (frequency>5% in controls) (Table 3). These common haplotypes were similar in frequencies to those found in the HapMap analysis, and the additional SNP included in this study, rs1041569 (T>A -2701), did not further discriminate between haplotypes. The threefold mutated haplotype, -2841*C/-2701*A/-871*T, which would have theoretically a cumulative high risk was not associated to SLE susceptibility. Indeed, the BAFF haplotype's frequencies distribution was quite comparable between SLE patients and controls (Table 3, global p-value=0.866). Again, haplotype analysis did not reveal any effect on disease presentation or on autoantibodies production and DCA. Equally, BAFF haplotype distributions did not influence the serum s-BAFF concentrations.

Discussion

Recent evidence has ignited the interest of a central role that B-cell holds in autoimmunity, not only by producing autoantibodies but also by the fact that B-cells intervene upstream in the pathophysiology of systemic autoimmune conditions [19], mainly SLE. This emphasizes the magnitude of the discovery of BAFF as a key player in B-cell survival and activation. Nevertheless, polymorphisms in BAFF gene were rarely investigated in SLE.

Haplotype*	Controls n=152	SLE n=124	p
T/A/C	0.39	0.35	0.32
C/A/T	0.21	0.25	0.44
T/A/T	0.16	0.14	0.49
T/T/T	0.13	0.09	0.44
C/A/C	0.04	0.05	0.97
C/T/T	0.02	0.05	0.16
T/T/C	0.009	0.01	0.51
C/T/C	0.003	0.01	0.24
Global test: $\chi 2$=3.19, df=7, p=0.866			

*Haplotype order: -2841 T>C /-2701 T>A/-871 C>T

Table 3: Combined haplotype (-2841 T>C/-2701 T>A/-871 C>T) frequencies in SLE patients and controls.

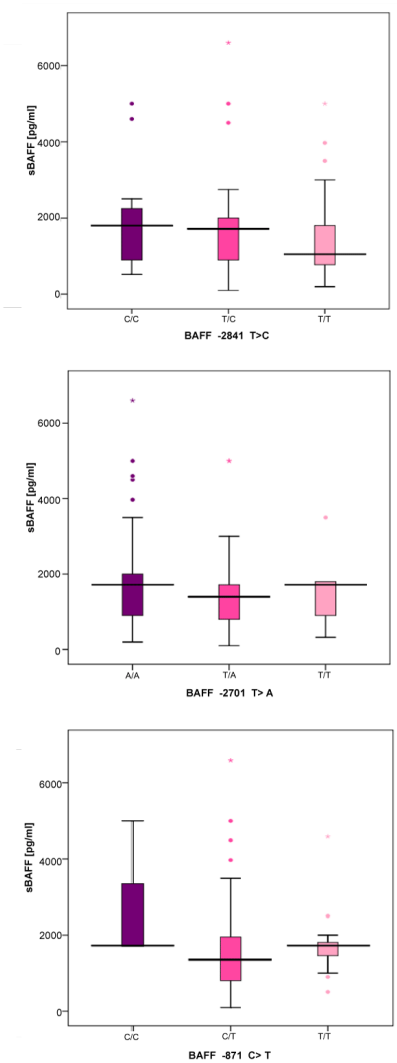

Figure 3: Analysis of s-BAFF levels according to BAFF SNPs.

In the present study we investigated genetically and quantitatively the impact of BAFF on SLE disease-susceptibility, activity (SLEDAI), several clinical manifestations and SLE-related biological features. The rs1041569 (T>A -2701) and rs9514828 (C>T -871) SNPs were not associated to SLE predisposition in our patients. This result corroborates those found in prior studies realized in Japanese [13] and Caucasian [20] populations. However, in a recent study performed in Egyptian SLE patients both -2701*C and -871*T mutated alleles were significantly associated with disease susceptibility, both p-values<10^{-3} [21]. This inedited unique association has to be considered with caution. In fact the Egyptian cohort involved only 50 patients and 30 controls, which is few for a common disease like SLE. Likewise, even if

our study included a larger sample (124 patients and 152 controls) the association of the -2841*C allele with SLE predisposition hadn't been reported before in previous studies [13,20,21], hence it has to be confirmed in independent cohorts and with family-studies. The low OR (1.47) indicates that even if the -2847*C allele could confer an increase in relative risk to SLE, its part within the whole genetic background would be too weak and can be missed in association studies. Besides, the significant correlation between the -2841*C allele and the presence of anti-dsDNA antibodies gives a clue about how this SNP would contribute into SLE pathophysiology. Indeed, by promoting this autoantibody production, the rs9514827 (T>C -2841) can initiate and/or aggravate the SLE-related tissue lesions through a possible increase in BAFF's expression.

A small meta-analysis for the three SNPs in the BAFF gene (T>C -2841, T>A -2701 and C>T -871) was performed with the Mantel-Haenszel method as a fixed-effects model test using the data of the three available studies [13,20,21] (Table 4). There were no significant associations for the T>C -2841 and the C>T -871 SNPs. Inversely, the combined model showed a significant correlation between the -2701*A mutated allele and SLE occurrence; p=0.0000032 OR (95% CI)=1.81 [1.41-2.38]. The weight of this association is mostly carried by the Egyptian cohort [21]. Indeed, the difference in frequencies of the -2701*A allele between patients and controls (0.66 vs 0.18, OR=9.91) is suspiciously and very strangely too much big for one SNP taken alone, and it even surpassed HLA estimated weight in SLE predisposition. Eventually, Egyptian and Tunisian populations share mostly the same genetic background, therefore such discrepancies in the observed results between the two studies is strange. Consequently, as the Egyptian cohort was too small (50 patients and 30 controls), the significant differences found has to be confirmed in a larger sample.

Author	Year	Population	Controls	Patients	*p*	OR (95% CI)
-2841 T>C						
Eilertsen [20]	2011	Norwegian	110	100	0.71	1.08 [0.71-1.63]
Zayed [21]	2013	Egyptian	30	50	0.46	1.33 [0.58-3.12]
Present study	2014	Tunisian	152	124	**0.013**	**1.57 [1.08-2.29]**
Combined fixed-effects model			292	274	0.058	1.27 [0.98-1.64]
-2701 T>A						
Eilertsen [20]	2011	Norwegian	110	100	0.076	1.62 [0.91-2.87]
Zayed [21]	2013	Egyptian	30	50	**>10^{-7}**	**9.91 [4.28-23.43]**
Present study	2014	Tunisian	152	124	0.71	0.92 [0.59-1.46]
Combined fixed-effects model			292	274	**0.0000032**	**1.83 [1.41-2.38]**
-871 C>T						
Kawazaki [13]	2002	Japanese	227	156	0.99	1 [0.74-1.35]
Eilertsen [20]	2011	Norwegian	110	100	0.27	0.81 [0.54-1.21]
Zayed [21]	2013	Egyptian	30	50	**10^{-7}**	**7.83 [3.27-19.23]**
Present study	2014	Tunisian	152	124	0.86	1.03 [0.72-1.46]
Combined fixed-effects model			519	430	0.29	1.1 [0.92-1.33]

Table 4: Meta-analysis of the three published studies of BAFF SNPs and SLE susceptibility.

Just like it had been previously related in other studies [14,20,22], we found a strong linkage disequilibrium between the three BAFF promoter SNPs. But, none of the observed common haplotypes was correlated to either disease-susceptibility, or activity, or SLE-related clinical and biological features. That was similarly the case in Norwegian SLE patients [20], while in the Egyptian study [21] haplotype analysis was not available. As mentioned before the BAFF*C/A/T (-2841/-2701/-871) was predictive for Ro/La autoantibody production in Norwegian primary SS patients [11], whereas in the present study we were not able to replicate this finding. In fact, the non-association of BAFF SNPs with SS specific antibodies can be explained by the fact that SLE-secondary SS is rather a different entity from the primary SS in both pathophysiological and serological meanings.

In order to evaluate BAFF promoter SNPs impact on serum s-BAFF levels we performed an ELISA test using anti-BAFF monoclonal antibody. As expected serum s-BAFF levels were drastically and hugely higher in patients than in controls, thus corroborating the overwhelming majority of published findings [23-25]. Moreover, s-BAFF level was significantly related to anti-dsDNA antibodies occurrence, therefore agreeing with data in literature [26]. This close correlation was established within a prospective investigation in which during the relapse, following the anti-CD20 B-cell depletion, subsequent B-cell repopulation was marked by a significant increase in BAFF levels and anti-dsDNA antibodies positivity [26]. BAFF transgenic mice develop SLE-like condition with production of anti-dsDNA antibodies [27], and the blockade of its specific receptor BAFF-R was enough to reduce significantly those autoantibodies incidence [28]. All these findings taken together lead to an ineluctable

conclusion: BAFF is directly involved in anti-dsDNA antibody synthesis through maintenance and activation of DNA-self-reactive B-cells. Interestingly, in the present cohort, BAFF levels were significantly higher in males than in females, which suggest that by lacking hormonal risk to develop autoimmunity, SLE occurrence in men might require more BAFF secretion to activate self-reactive B-cells.

Two studies performed in Egyptian SLE patients reported significant associations of serum s-BAFF levels with SLAM (Systemic Lupus Activity Measure) [24] and SLEDAI [25]. While, and strangely, in the present study the serum s-BAFF level did not match with SLE disease-activity which had been reported in several case-report studies [16,20,23,29,30]. Inversely, functional studies of BAFF mRNA levels confirmed the close correlation with SLE activity [30,31]. This discrepancy between serum s-BAFF and BAFF mRNA in predicting SLE activity may be due to a relative lack of correlation between these two parameters: first, the serum s-BAFF was not associated either to BAFF expression locally in damaged tissues or to the stage of B-Cell infiltration [32]. Second, as there are several s-BAFF isoforms (homotrimer, Heterotrimer with APRIL, Δ3-BAFF, Δ4-BAFF) the monoclonal antibody used to trap s-BAFF in ELISA may not recognize all of them, mostly the truncated isoforms (Δ3-BAFF and Δ4-BAFF). Third, some SLE patients develop anti-BAFF autoantibodies *in vivo* [30] which block sterically the epitopes recognized by detecting antibodies in the *in vitro* ELISA. In this case, there will be a bias in measurement of s-BAFF levels that would be spuriously reduced leading to a masking of BAFF over-expression, and by corollary, to hiding correlations with SLE activity.

In the current study, assessment of genetic impact on serum s-BAFF levels failed to find any associations with the whole three investigated promoter SNPs (T>C -2841, T>A -2701 and C>T -871) whether taken alone or combined in haplotypes. This was evenly the case in Caucasoid [20], Japanese [13] and American [23] populations. Actually, if the serum s-BAFF level was not informative of BAFF's over-expression (elevated BAFF mRNA), it could not be correlated to BAFF genetic variants especially those who are potentially increasing its promoter activity. Thus, in order to estimate the real impact of promoter SNPs on BAFF, genetic studies should focus on local BAFF expression in damaged tissues such as kidney in case of lupus nephritis or in skin biopsies.

Conclusion

In Tunisian, the rs9514827 (T>C -2841) SNP in the BAFF gene promoter seems to be related to SLE susceptibility and anti-dsDNA antibodies occurrence. Even if serum s-BAFF levels were significantly higher in SLE patients and in anti-dsDNA antibody positive sera, it did not seem to be correlated with disease activity or the occurrence of lupus nephritis. Again, regulatory genetic polymorphisms were not correlated to s-BAFF which is in agreement with the reported discrepancies between BAFF mRNA and s-BAFF levels. Therefore, we are intending to study the impact of the BAFF promoter SNPs on its local expression in the damaged tissues, particularly in kidney biopsies in case of lupus nephritis and skin biopsies.

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Competing Interests

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Authors' Contribution

Pr Gorgi Yousr proposed the study and wrote the first draft. Tarak Dhaouadi and Imen Sfar analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. Pr Gorgi Yousr is the guarantor of the integrity of this study.

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