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*Chromosome 2q33*genetic polymorphisms in Tunisian endemic pemphigus foliaceus

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Abstract

Background: Several studies have suggested that polymorphisms within genes encoding T-lymphocyte immune regulating molecules: *CD28*, *CTLA-4*, and *ICOS*, may alter the signaling process and subsequently could be involved in susceptibility to a broad spectrum of autoimmune diseases.

Methods: This study aimed to replicate associations between common polymorphisms in the 2q33.2 cluster and susceptibility to pemphigus foliaceus (PF) in the Tunisian population. We investigated seven polymorphisms: rs3116496 and rs1879877 (*CD28*), rs231775, rs3087243, and $(AT)_n$ repeat (*CTLA4*); rs11889031 and rs10932029 (*ICOS*) in a case–control study which enrolled 106 Tunisian PF patients and 205 matched healthy controls.

Results: We confirmed the associations with *CTLA4*($(AT)_{13}$, $p = 0.00137$, OR = 3.96 and $(AT)_{20}$, $p = 0.008$, OR = 5.22; respectively) and *ICOS* genes (rs10932029>CT, $p = 0.034$, OR = 2.12 and rs10932029>TT, $p = 0.04$ and OR = 0.41).

Conclusion: Our results indicate that susceptibility to PF is located in the proximal and the distal 3′ flanking region of the *CTLA4*/*ICOS* promoter. These findings may open avenues to the treatment of patients with biological drugs targeting CTLA4/ICOS molecules, in a personalized manner to achieve more effective treatment.

KEYWORDS

2q33 polymorphisms, genetic susceptibility, pemphigus foliaceus, Tunisia

1 | INTRODUCTION

Pemphigus foliaceus (PF) is a chronic autoimmune blistering skin disease characterized by acantholysis most likely associated with downstream events following the binding of desmoglein-1(Dsg1) specific IgG pathogenic auto-antibodies (-Abs) (Lee et al., 2009). PF develops as either sporadic disease occurring all over the world or in an endemic form. The latter is found mainly in South America and North Africa (especially in Tunisia) (Abida, Kallel-Sellami, et al., 2009; Bastuji-Garin et al., 1995). Pemphigus is associated with severe morbidity affecting the quality of life of young subjects and considerable mortality. Pemphigus therapies aim to improve symptoms by reducing serum autoantibodies, either directly or through generalized immune suppression

TABLE 1 Characteristics of study populations

Features	PF Patients	Endemic group	Sporadic group	Controls
Number	106	36	70	205
Mean age	35 [18-84]	32 [18-60]	37 [19-84]	38 [14-73]
Origin	Center/south	3 localities[a] South	Other regions Center/south	Center/south
Sex ratio M/F	1/14	0/36	1/9	1/9

[a]Endemic localities in the south of Tunisia (Abida, Kallel-Sellami, et al., 2009).

(Committee for Guidelines for the Management of Pemphigus Disease et al., 2014; Hertl et al., 2015). Systemic corticosteroids combined with adjuvant therapies were usually used as the first-line treatment for *de novo* patients and significantly improved survival of the patients. During recent years, rituximab (RTX), a monoclonal anti-CD20 antibody that targets $CD20^+$ B cells, has being increasingly used (Joly et al., 2017). However, some evidence of exacerbation of disease was found following RTX (Feldman, 2015).

Increasingly, several studies have highlighted the advantages of the immune checkpoint modulators to conversely treat autoimmunity (Ellebrecht et al., 2016). CD28/CTLA-4 system functions as a rheostat that can tune T cell activation up or down. These receptors have both stimulatory (CD28, ICOS) and inhibitory roles (CTLA-4) in T-cell function. Based on its crucial role in immunological homeostasis, CTLA4/CD28 pathways are of considerable interest in the treatment of cancer and autoimmune diseases (AID) using antibodies and fusion proteins (Rowshanravan, Halliday, & Sansom, 2018). The chromosome 2q33 harbor the cluster of T-lymphocyte immune regulating genes: T-cell antigen CD28 (*CD28#*186760), Cytotoxic T Lymphocyte-Associated Protein 4 (*CTLA-4#*123890), and Inducible T-cell Costimulator (*ICOS#*604558). It has become one of the main genes of research interest in association studies (Scalapino & Daikh, 2008). Several studies have suggested that polymorphisms within genes encoding those molecules may alter the signaling process and subsequently be involved in susceptibility to a broad spectrum of AID (Kim et al., 2011; Mahmoudi et al., 2020; Zouidi et al., 2014).

Various investigations reported the involvement of genetic factors in the emergence of PF. Thus, several pathways have been implicated in the etiopathogenesis of the disease (Aoki, Rivitti, & Diaz, 2015; Masmoudi, Abida, Masmoudi, & Turki, 2019; Tron et al., 2005). Exiting results from more recent genetic studies have suggested the genes encoding the 2q33 molecules as a possible pathway in the pathogenesis of the Brazilian endemic PF (Dalla-Costa, Pincerati, Beltrame, Malheiros, & Petzl-Erler, 2010; Narbutt et al., 2010), although previous studies on the same disease have failed to detect any association (Fernández-Mestre et al., 2009; Pavoni, Cerqueira, Roxo, & Petzl-Erler, 2006; Pincerati, Dalla-Costa, & Petzl-Erler, 2010). Polymorphisms of 2q33 genes have not been analyzed in Tunisian endemic PF disease to date. The identification of 2q33 polymorphisms associated with pemphigus before initiation of treatment may open new perspectives for the treatment of patients in a personalized manner to achieve more effective treatments. Thus, it is not surprising that biological drugs show different efficacy in patients according to their particular genetic profiles.

This study aimed to replicate associations between common polymorphisms in the 2q33.2 cluster and susceptibility to PF in a Tunisian case/control study. Six polymorphisms were analyzed in DNA samples from 106 Tunisian PF patients and 205 matched healthy controls: rs3116496 and rs1879877 (*CD28*), rs231775, $(AT)_n$ repeat, rs3087243 (*CTLA-4*); rs11889031 and rs10932029 (*ICOS*).

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

Informed consent was obtained from all participants. The study received approval by the Human Research Ethics Committee of the Habib Bourguiba University Hospital of Sfax (protocol number of the ethical committee, 4/12).

2.2 | Samples

This genetic retrospective study is a case–control study performed in the Tunisian population since 2002. Patients have been recruited at the Department of Dermatology in the Hedi Chaker University Hospital of Sfax, Tunisia. One hundred six (106) PF patients were matched by age (±5 years), gender, and geographical origin to two hundred five (205) healthy controls (HC) (Table 1). The diagnosis of PF was confirmed by clinical presentation, histopathology (acantholysis in the upper epidermis either in the granular layer or immediately below with subcorneal bullous formation), direct immunofluorescence (IgG and C3 deposits most often located on the whole epidermis and less frequently predominant in the upper layers of the epidermis), indirect immunofluorescence (IgG Abs directed against the epithelial cell surface), and ELISA test for circulatory anti-Dsg1 Abs that was positive for all

patients (Kershenovich, Hodak, & Mimouni, 2014). PF patients exhibit different clinical types of the disease: (33/106) pemphigus erythematosus, and (64/106) the more extensive type referred to as generalized Pemphigus. Pemphigus herpetiformis with anti-Dsg1 auto-Abs was also described (9/106).

Control subjects were recruited among healthy blood donors with no signs of autoimmune disorders.

The patient group included 8 men and 98 women, with a mean age of 35 years ranging from 18 years to 84 years. All patients originate from the center and south of Tunisia. The control group included 21 men and 180 women, with a mean age of 39 years ranging from 14 years to 73 years. Related patients and/or HC were excluded from the study (Table 1).

2.3 | Selection of polymorphisms

To select the most representative genetic variations, tag polymorphisms were selected using the genotyping data from the CEU available from the International HapMap project and according to their association with the susceptibility to other AID. Generally, polymorphisms were selected for potentially functional relevance. Thus, polymorphisms could influence gene expression through changes in promoter activity, the stability of messenger RNA, and protein functioning (Shastry, 2009).

rs1879877 (promoter) and rs3116496 (intron3) (*CD28*; NG_029618.1), rs11889031 (in the downstream regulatory region (−693GA)), and rs10932029 (+173: which covers the promoter of the *ICOS* gene (NG_011586.1) and the 3′ region of the *CTLA4* gene (NG_011502.1) were selected by these means (Table 2). Also, three polymorphisms ranging from the promoter region (rs231775 AG49) to the 3′ UTR of the *CTLA4* gene (the rs3087243 named CT60 and the (AT) repeat) were analyzed.

2.4 | Genotyping of 2q33.2 SNPs

Genomic DNA was extracted from whole blood samples using a standard proteinase K digestion and phenol/chloroform extraction procedure. Genotyping was performed using the PCR-RFLP method for all SNPs.

Primers were designed using primer3 software (http://primer3.ut.ee/). The primers are illustrated in Table 2. Classic and touchdown PCR reactions were performed in a thermal cycler (Simpli Amp Thermal Cycler, Thermo Fisher®). Reactions were cycled 30 times with denaturation at 95°Cfor 35 s, hybridization for 35 s, and extension at 72°C for 45 s. The PCR amplification was carried out in a volume of 25 µl including 1x buffer, 2 mM MgCl2, 0.2–0.4 µmol of each primer (Invitrogen®, CA, USA), 0.12 mMdNTP (Invitrogen®,

TABLE 2 Primary information of genotyped polymorphisms in the 2q33 chromosome region

Gene #OMIM	Polymorphism	Base change	localization	Primers	Annealing temperature (°C)	Enzyme
CD28#186760	rs1879877	c.-1198 C>A	5′near gene	F:5′-CAAAAGCCCTTGGTGTCTC-3′ R:5′-AGATACTGACGTAGGTGCCG-3	59	EarI
	rs3116496	c.17 + 3 T>C	Intron3	F:5′CGGACCTTCTAAGCCCTTTT-3′ R:5′CCTACTCAAGCATGGGGAAA-3	61	Aci 1
CTLA-4#123890	rs231775	c.+49A>G	Exon1	F:5′-GCTCTACTTCCTGAAGACCT-3′ R:5′-AACCCAGGTAGGAGAAACAC-3′	Touch down 60-50	BbvI
	(AT)n[a]	c.642AT(8_33)	3′UTR	F:5′-Fam-GATGCTAAAGGTTGTATTGC-3′ R:5′-TGGTGTATTAGTGTCCTG-3′		
	rs3087243	c.+6230G>A CT60	3′UTR	F:5′-AGGGGAGGTGAAGAACCTGT-3′ R:5′-CAAATAATGCTTCATGAGTCAGC-3′	58	TaiI
ICOS#604558	rs11889031	c.-693G/A	promotor	F:5′-CAAACTGAGAAGCGAGAG-3′ R: 5′-ATAAGTTCTAGAGCTCAGGG-3′	58	XmnI
	rs10932029	c.+173T/C	Intron1	F:5′-CCTCTGGTATTTCTTTCTCTTC-3′ R:5′-ACAGGTAACTCCAAGCAG-3′	56	DdeI

Note: CD28 gene (NG_029618.1), *ICOS* gene (NG_011586.1) and CTLA4 gene (NG_011502.1).

[a]Primers described in (Hadj Kacem et al., 2001).

CA, USA),1 U Taq polymerase (Invitrogen®, CA, USA), and 50 ηg of DNA template.

Restriction enzymes were selected using the NEBcutter software (http://nc2.neb.com/NEBcutter2/). Enzymatic digestion was performed in a total of 10 µl mixture reaction containing 1x buffer, 0.1x BSA, and 2 U restriction enzyme (Thermo Fisher®, MA, USA), according to the instructions of the manufacturer.

2.5 | Genotyping of the microsatellite marker

The genotype of the *CTLA-4*$(AT)_n$ microsatellite was identified using PCR and fragment size determination. Primers were designed as described previously (Hadj Kacem, Bellassoued, Bougacha-Elleuch, Abid, & Ayadi, 2001)and based on the genomic reference sequence and labeled with the 6-Carboxyfluorescein fluorescent dye (FAM). Briefly, the PCR amplification was carried out in a volume of 10 µl including 1x buffer, 2.5 pmol of each primer (Invitrogen®, CA, USA), 10 mMdNTP (Invitrogen®, CA, USA), 1 U AmpliTaq Gold™ DNA Polymerase (Applied Biosystems™, CA, USA), and 1 ηg of DNA template. Genotypes were assigned on basis of the sizes of the PCR products by capillary electrophoresis in ABI prism DNA sequencer after denaturation (Perkin Elmer®, CT, USA)and output file was analyzed using Gene Scan software analysis.

2.6 | Statistical analysis

A case–control analysis was performed using SHESIS software (http://analysis.bio-x.cn) for each SNP and haplotype. Hardy–Weinberg equilibrium (HWE) was assessed in controls using a chi-square test with one degree of freedom. A threshold was regarded to indicate the deviation from HWE. Comparisons between frequencies in the patient and control population samples were performed by analysis of contingency tables by the chi-square test of independence. The strength of the associations was estimated by the odds ratio (OR) with 95% confidence intervals (CI). The *p*-value of 0.05 was adopted as the significance limit for all statistical tests. The linkage disequilibrium (LD) coefficients $D' = D/Dmax$ and r^2 values for the pair of the most common alleles at each site were also estimated, and high values of LD were defined as $r^2 > 0.33$ and $D' > 0.7$.

3 | RESULTS

The genotypic and allelic distributions of the *2q33.2* cluster tag polymorphisms as well as their associations with the risk to PF are shown in Table 3. Genotype frequencies were in HWE in patients and control population samples respectively.

3.1 | *CD28* gene

Concerning the *CD28*gene, the rs1879877>AC polymorphism seems to be slightly associated with the PF's risk in the whole population. In fact, the heterozygote AC genotype was more expressed in patients (39.4%) compared to HC (28%) ($p = 0.05$, OR = 1.67, 95% CI 1–2.83). Considering the PF-stratified analysis, thers1879877 AC genotype exhibited a susceptibility role to PF ($p = 0.035$, OR = 1.99 95% CI 1.04–3.78) contrary with the CC homozygote genotype which was quite more expressed in controls (67%) than in patients (53%) ($p = 0.06$, OR = 0.56, 95% CI 0.3–1.05).

Likewise, the increased expression of the rs3116496CT genotype observed in the patient group (33.7%) compared to HC (22.7%) suggests its susceptible role in PF ($p = 0.05$, OR = 1.73, 95% CI 1–3). No association was found after disease-stratified analysis.

3.2 | *CTLA4* gene

Considering the total samples and evaluating the microsatellite (AT) repeat in the *CTLA4* gene, 21 different alleles composed by repeats $(AT)_7$-$(AT)_{31}$ ranging from 185 to 229 bp were revealed. In both patients and control groups, the most common alleles were $(AT)_7$ (48.7% and 55% respectively). It was found that $(AT)_{13}$ and $(AT)_{20}$ alleles increase the risk of PF among the Tunisian patients ($p = 0.00137$ with OR = 3.96 95% CI [1.63–9.64]) and $p = 0.008$ with OR = 5.22 95% CI [1.41–19.28] respectively).

AT microsatellite alleles and genotypes frequencies were investigated in endemic and sporadic patients' groups and their respective matched control groups separately to look for possible stronger differences and/or new associations. The *CTLA-4* AT repeat seems to be closely associated with the sporadic PF's risk. In fact, the AT_{20} allele was significantly overexpressed in patients (8.2%) compared to HC (0.6%) ($p = 0.0017$; OR = 13.6; 95% CI 1.67–110). No association was reported in the endemic group.

There were no associations between the distribution of AT repeat genotypes and the disease in the endemic group.

The allelic and genotypic distributions of the studied SNPs were quite similar between patients and HC groups. There was a lack of association between the SNPs in the*CTLA4* gene, including rs231775 and rs3087243 and PF risk in the Tunisian population.

TABLE 3 Genotype and allele frequencies of 2q33 polymorphisms in pemphigus foliaceus patient's groups and their relative matched healthy controls

	Whole population				Endemic group				Sporadic group			
Gene/polymorphism	Case n = 106 (%)	Controls n = 205 (%)	*p*	OR 95% CI	Case N = 36 (%)	Controls N = 76 (%)	*p*	OR 95%CI	Case N = 70 (%)	Controls N = 129 (%)	*p*	OR 95% CI
CD28>rs1879877												
A	45 (22.7)	60 (18.3)	0.2		12 (18.2)	20 (17.2)	0.8		33 (25)	40 (18.9)	0.2	
C	153 (77.3)	268 (81.7)			54 (81.8)	96 (82.8)			99 (75)	172 (81.1)		
AA	3 (3)	7 (4.3)	0.6		1 (3)	2 (3.4)	0.9		2 (3)	5 (4.7)	0.6	
AC	39 (39.4)	46 (28)	0.05	1.67 [1-2.83]	10 (30.3)	16 (27.6)	0.7		29 (43.9)	30 (28.3)	0.035	1.99 [1.04-3.78]
CC	57 (57.6)	111 (67.7)	0.09		22 (66.7)	40 (69)	0.8		35 (53)	71 (67)	0.06	
CD28>rs3116496												
C	38 (18.8)	53 (16.3)	0.4		9 (13.6)	17 (13.9)	0.9		29 (21.3)	36 (17.6)	0.4	
T	164 (81.2)	273 (83.7)			57 (86.4)	105 (86.1)			107 (78.7)	168 (82.4)		
CC	2 (2)	8 (4.9)	0.2		0	3 (4.9)	0.2		2 (2.9)	5 (4.9)	0.4	
CT	34 (33.7)	73 (39.9)	0.05	1.73 [1-3]	9 (27.3)	11 (18.1)	0.3		25 (36.8)	26 (25.5)	0.1	
TT	65 (64.4)	118 (72.4)	0.1		24 (72.7)	47 (77)	0.2		41 (60.3)	71 (69.6)	0.2	
CTLA4>rs231775												
A	117 (68.8)	218 (69.9)	0.8		49 (76.6)	81 (72.3)	0.5		68 (64.2)	137 (68.5)	0.4	
G	53 (31.2)	94 (30.1)			15 (23.4)	31 (27.7)			38 (35.8)	63 (31.5)		
AA	37 (43.5)	76 (48.7)	0.4		18 (56.2)	28 (50)	0.5		19 (35.8)	48 (48)	0.15	
AG	43 (50.6)	66 (42.3)	0.2		13 (40.6)	25 (44.6)	0.7		30 (56.6)	41 (41)	0.06	
GG	5 (5.9)	14 (9)	0.4		1 (3.1)	3 (5.4)	0.6		4 (7.5)	11 (11)	0.1	
CTLA4>ATn												
7	78 (48.7)	131 (55)	0.2		33 (53.2)	51 (60.7)	0.4		45 (45.9)	80 (51.9)	0.3	
10	2 (1.3)	0 (0)	0.08		1 (1.6)	0	0.2		1 (1)	0	0.2	
11	1 (0.6)	3 (1.3)	0.5		0	2 (2.4)	0.2		1 (1)	1 (0.6)	0.7	
12	0	4 (1.7)	0.1		0	0	-		0	4 (2.6)	0.1	
13	6 (3.7)	2 (0.8)	0.042	4.6 [0.92-23]	3 (4.8)	1 (1.2)	0.2		3 (3.1)	1 (0.6)	0.1	
14	3 (1.9)	1 (0.4)	0.1		0	1 (1.2)	0.4		3 (3.1)	0	0.2	
15	3 (1.9)	4 (1.7)	1		0	1 (1.2)	0.4		3 (3.1)	3 (1.9)	0.6	
16	23 (14.4)	30 (12.6)	0.8		6 (9.7)	6 (7.1)	0.6		17 (17.3)	24 (15.6)	0.7	

TABLE 3 Continued

	Whole population				Endemic group				Sporadic group			
Gene/polymorphism	Case n = 106 (%)	Controls n = 205 (%)	*p*	OR 95% CI	Case N = 36 (%)	Controls N = 76 (%)	*p*	OR 95%CI	Case N = 70 (%)	Controls N = 129 (%)	*p*	OR 95% CI
17	8 (5)	12 (5)	0.7		3 (4.8)	1 (1.2)	0.2		5 (5.1)	11 (7.1)	0.5	
18	6 (3.7)	13 (5.5)	0.2		2 (3.2)	5 (6)	0.4		4 (4.1)	8 (5.2)	0.7	
19	2 (1.3)	3 (1.3)	0.8		1 (1.6)	1 (1.2)	0.8		1 (1)	2 (1.3)	0.8	
20	10 (6.2)	3 (1.3)	0.008	5.22 [1.41-19.28]	2 (3.2)	2 (2.4)	0.8		8 (8.2)	1 (0.6)	0.0017	13.6 [1.67-110]
21	5 (3.1)	6 (2.5)	0.8		3 (4.8)	2 (2.4)	0.4		2 (2)	4 (2.6)	0.8	
22	2 (1.3)	6 (2.5)	0.2		1 (1.6)	5 (6)	0.2		1 (1)	1 (0.6)	0.7	
23	1 (0.6)	1 (0.4)	0.7		0	0	-		1 (1)	1 (0.6)	0.7	
24	1 (0.6)	4 (1.7)	0.2		1 (1.6)	2 (2.4)	0.7		0	2 (1.3)	0.2	
25	1 (0.6)	6 (2.5)	0.1		0	4 (4.8)	0.08		1 (1)	2 (1.3)	0.8	
26	4 (2.5)	3 (1.3)	0.3		2 (3.2)	0	0.09		2 (2)	3 (1.9)	0.9	
27	2 (1.3)	3 (1.3)	0.8		2 (3.2)	0	0.09		0	3 (1.9)	0.1	
28	2 (1.3)	2 (0.8)	0.6		2 (3.2)	0	0.09		0	2 (1.3)	0.2	
31	0	1 (0.4)	0.3		0	0	-		0	1 (1.6)	0.4	
CTLA4>rs3087243												
C	105 (54.1)	189 (51.4)	0.5		34 (51.5)	65 (47.1)	0.5		71 (55.5)	124 (53.9)	0.7	
T	89 (45.9)	179 (48.6)			32 (48.5)	73 (52.9)			57 (44.5)	106 (46.1)		
CC	29 (29.9)	54 (29.3)	0.9		9 (27.3)	17 (24.6)	0.7		20 (31.2)	37 (32.2)	0.9	
CT	47 (48.3)	81 (44)	0.4		16 (48.5)	31 (44.9)	0.7		31 (48.4)	50 (43.5)	0.5	
TT	21 (21.6)	49 (26.6)	0.3		8 (24.2)	21 (30.4)	0.5		13 (20.3)	28 (24.3)	0.5	
ICOS>rs11889031												
C	104 (76.5)	150 (82.4)	0.2		36 (72)	50 (80.6)	0.2		68 (79.1)	100 (83.3)	0.4	
T	32 (23.5)	32 (17.6)	0.3		14 (28)	12 (19.4)			18 (20.9)	20 (16.7)		
CC	39 (57.4)	60 (65.9)	0.2		13 (52)	19 (61.3)	0.5		26 (60.5)	41 (68.3)	0.4	
CT	26 (38.2)	30 (33)	0.5		10 (40)	12 (38.7)	0.9		16 (37.2)	18 (30)	0.4	
TT	3 (4.4)	1 (1.1)	0.2		2 (8)	0 (0)	0.1		1 (2.3)	1 (1.7)	0.8	
ICOS>rs10932029												
C	23 (13.9)	28 (8.6)	0.07		11 (18.3)	8 (6.6)	0.014	3.2 [1.21-8.44]	12 (11.3)	20 (9.8)	0.6	

TABLE 3 Continued

Gene/polymorphism	Whole population				Endemic group				Sporadic group			
	Case n = 106 (%)	Controls n = 205 (%)	*p*	OR 95% CI	Case N = 36 (%)	Controls N = 76 (%)	*p*	OR 95%CI	Case N = 70 (%)	Controls N = 129 (%)	*p*	OR 95% CI
T	143 (86.1)	298 (91.4)			49 (81.7)	114 (93.4)			94 (88.7)	184 (90.2)		
CC	2 (2.4)	4 (2.5)	0.9		2 (6.7)	2 (3.3)	0.4		0	2 (2)	0.3	
CT	19 (22.4)	20 (12.3)	0.034	2.12 [1.06-4.25]	7 (23.3)	4 (6.6)	0.021	4.34 [1.16-16.24]	12 (22.6)	16 (15.7)	0.3	
TT	62 (74.4)	139 (85.3)	0.04	0.41 [0.26-0.98]	21 (70)	55 (90.2)	0.014	0.25 [0.08-0.8]	41 (77.4)	84 (82.4)	0.5	

Note: CD28 gene (NG_029618.1), *ICOS* gene (NG_011586.1) and CTLA4 gene (NG_011502.1).

3.3 | *ICOS* gene

Regarding the *ICOS* gene, the rs10932029 CT genotype was significantly more frequent in PF patients than in controls in the total samples ($p = 0.034$, OR = 2.12 95% CI 2.12–4.25), suggesting a possible pathogenic role of rs10932029, contrary with the TT genotype which was more expressed in controls ($p = 0.04$, OR = 0.41 95% CI 0.26–0.89) suggesting its protective role against PF. Likewise, the same associations were observed only in the endemic patient group compared to their relative HC ($p = 0.021$, OR = 4.35, 95% CI 1.16–16.24 and $p = 0.014$, OR = 0.25 95% CI 0.08–0.8 respectively). A significant increase in the rs10932029>C allele was observed in the patient group (18.3%) compared to HC (6.6%) ($p = 0.014$, OR = 3.2, 95% CI 1.21–8.44).

No association was found with rs118890311 also named −693 GA.

3.4 | 2q33 polymorphisms and PF features

On the other hand, chi-square analysis indicated a barely significant difference in gene frequencies between patient groups for both *CTLA4*>rs231775and *ICOS*>rs10932029. Indeed, the distributions of rs231775>AA and rs10932029>CC homozygous genotypes were more frequent in patients from endemic regions than in sporadic patients ($p = 0.06$ and $p = 0.057$ respectively). Moreover, the distribution of the rs10932029>CC genotype was associated with the different clinical types of the disease ($p = 0.04$). Absent in patients with generalized pemphigus, it was found in 4.5% of those with pemphigus erythematosus and 11% of herpetiformis pemphigus patients.

3.5 | Linkage disequilibrium (LD) and haplotype

The LD analysis among the patient group was conducted by pairwise comparison of the seven polymorphisms studied in the 2q33.2 chromosome region (see Figure 1).

When considering the total samples, the evidence of the existence of LD was revealed between the 5′UTR rs118890311 with rs10932029 located in +173 base from the ATG initial codon in *ICOS* gene (D′ = 0.99), indicating that the association observed with rs10932029 should be independent. LD values measured by D′ in disease-stratified groups highlight f than evidence that LD was more pronounced in the endemic patient's group. Thus, strong evidence for LD was revealed between the two *ICOS* gene SNPs: rs118890311 versus rs10932029 (D′ = 0.99). The latter was also in strong LD with *CD28* gene's studied SNPs (rs1879877 and rs3116496) and limited with the rs231775

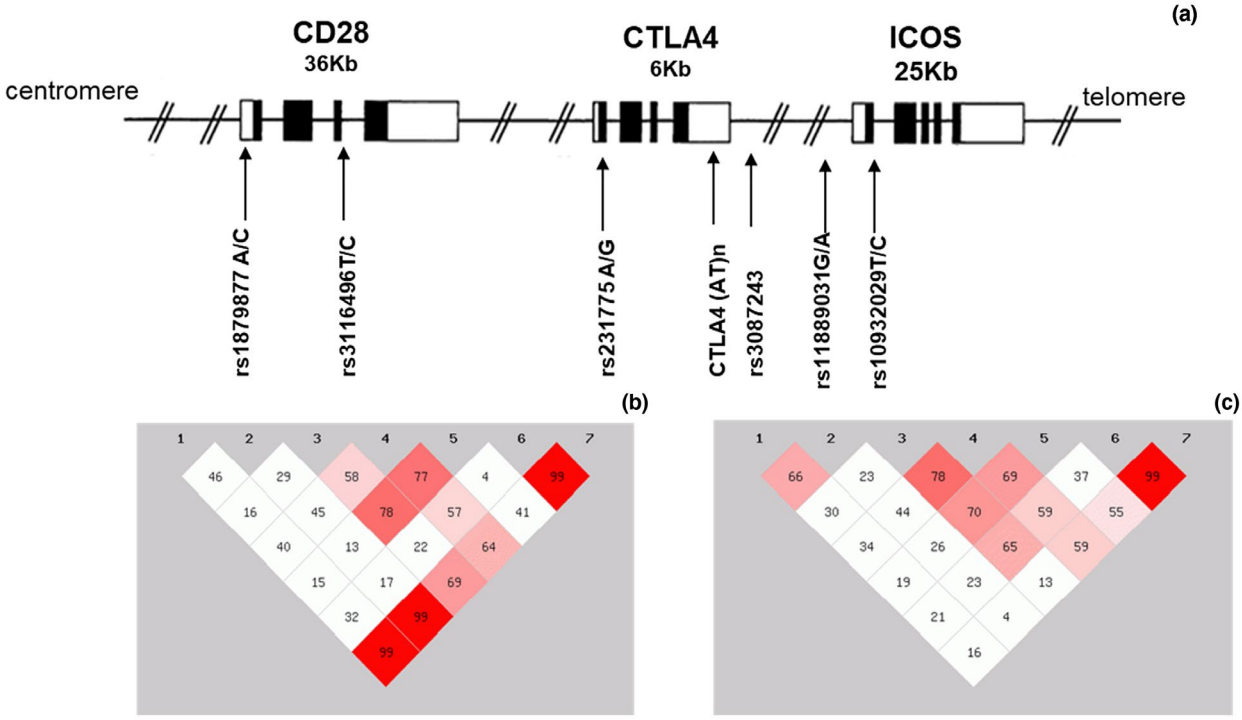

FIGURE 1 Overview and linkage disequilibrium (LD) on chromosome 2q33.2. Schematic structure of the 2q33.2 chromosome region *CD28* gene (NG_029618.1), *ICOS* gene (NG_011586.1) and CTLA4 gene (NG_011502.1) (a). Exons are depicted by black vertical lines. The positions of the seven polymorphisms are shown by the arrows. LD prime charts generated using SHEsis software summarize LD (D′) patterns among endemic (b) and sporadic (c) patient's groups. D′ > 0.7 is considered as a high value of LD

in *CTLA4* gene (D′ = 0.99, 0.99 and 0.69; respectively). Furthermore, the *CTLA4* 3′UTR polymorphism (rs3087243 also called CT60) was in LD with other *CTLA4* studied polymorphisms: rs231775 (D′ = 0.78) situated at +49 bases from the promoter of *CTLA4* gene and the AT repeat polymorphism in the 3′UTR region (D′ = 0.77). In the sporadic group; only the LD noted between the rs118890311 and rs10932029 in the *ICOS* gene (D′=0.99) was maintained. The LD between *CTLA4*-rs3087243 and other *CTLA4* polymorphisms was limited (rs231775 (D′ = 0.70) and (AT)n repeat (D′ = 0.69).

On the other hand, the 2q33.2 haplotypes were assessed for the PF patient groups and their respective controls. Analysis of the rs1879877-rs3116496-rs231775-$(AT)_n$-rs3087243-rs11889031-rs10932029 haplotypes, by the SHEsis program, revealed distinct haplotypes (Table 4). Thus, the CTA7TCT haplotype was significantly more expressed in PF patients than in controls, in the total samples (35% vs. 19%: $p = 0.0001$, OR = 5.22[2.16–12.6]) and the endemic group (31.8% vs. 19%: $p = 0.027$, OR = 3.39 [1.28–10.19]. Whereas, the CTA7CCT haplotype was shown to be a protector haplotype in the whole population ($p = 0.0003$, OR = 0.05 [0.008–10.19]) as well as in the sporadic group ($p = 0.002$).

Furthermore, and in the sporadic group, the CTG16CCT haplotype was more expressed in controls (8.6%) than in patients (1.6%) ($p = 0.028$, OR = 0.12 [0.0014–1.063]. The later was not associated with the disease in the total samples.

4 | DISCUSSION

Therapies targeting the T cell checkpoints pathways have been suggested as new opportunities for the treatment of AID. Identification of genetic profiles of checkpoints molecules gene's is essential to achieve more effective treatments. To underline the role of the genes involved in these pathways, we investigated common polymorphisms in the 2q33.2 chromosome region and susceptibility to PF in the Tunisian population.

For the *CD28* gene, rs3116496> CT and especially the rs1879877> AC genotypes seem to be slightly associated with the risk of PF. Indeed, and following our results, *CD28*>rs1879877 polymorphism seemingly does not influence susceptibility to PF in the Brazilian population (Dalla-Costa et al., 2010). Interestingly, a significant association was found between PF disease status and (CAA) microsatellite within the 3′UTR of the *CD28* gene (Dalla-Costa et al., 2010). Since, rs1879877 SNP of the *CD28* gene is located in the promoter region of the gene, its biological relevance is due to its role in the control of the level of expression of CD28 membrane protein and in doing so the increase or

TABLE 4 Haplotypes of genotyped polymorphisms on 2q33 chromosome region

	Haplotype[a]	Patients *N* (%)	Controls *N* (%)	*P*	OR	95% CI
Total samples	CTA7TCT	39 (35)	23 (19)	0.0001	5.22	[2.16-12.6]
	CTA7CCT	1 (1)	15 (13)	0.0003	0.05	[0.008–10.19]
Sporadic group	CTA7CCT	0 (0)	8 (10.5)	0.002	—	
	CTG16CCT	1 (1.6)	6 (8.6)	0.028	0.12	[0.0014–1.063]
Endemic group	CTA7TCT	15 (32)	8 (19)	0.027	3.39	[1.28–10.19]

[a]Haplotype analysis of studied polymorphisms:*CD28* gene (NG_029618.1): rs1879877 A/C and rs3116496 C/T; *CTLA4* gene (NG_011502.1): rs231775 A/G, (AT)n and rs3087243 and *ICOS* gene (NG_011586.1): rs11889031 C/T and rs10932029 C/T.

decrease in cellular responses related to CD28 (Kim et al., 2011). Furthermore, many studies showed that the CD28 receptor is involved in the control of the Th1/Th2 switch during the development and progression of AID like pemphigus vulgaris (PV), the other prototype of pemphigus (Kuchroo et al., 1995; Toto et al., 2000).

Evaluating polymorphisms in the *CTLA4* gene, our study indicated that $(AT)_{20}$ repeat within the 3′-UTR of the gene was associated with PF in southern Tunisia. However, no associations were reported between the rs231775 and rs3087243 of *CTLA4* gene polymorphisms. Our findings were concordant with previous findings regarding variation in the *CTLA4* gene in PF disease. Although SNPs within *CTLA4* are associated with a very wide range of inflammatory and AIDs (Gough, Walker, & Sansom, 2005), there was a lack of association between various SNPs in this gene, including rs231775 (AG49) and rs3087243 (CT60) and PF in different populations (Narbutt et al., 2010; Pavoni et al., 2006; Pincerati et al., 2010). On contrary, it was found that the rs231775 GG genotype and G allele increase the risk of PV (Fernández-Mestre et al., 2009). Consistent with the Brazilian population results, *CTLA4* $(AT)_n$ repeat is also associated with susceptibility to PF (Dalla-Costa et al., 2010). Furthermore, the *CTLA4* (AT)n microsatellite marker has been reported as associated with AIDs, such as systemic lupus erythematosus (Ahmed et al., 2001). This polymorphism is potentially important because it may be involved in the determination of mRNA stability, as suggested by several reports (Yanagawa, Hidaka, Guimaraes, Soliman, & DeGroot, 1995). It has been supposed that shorter alleles may be more stable and longer may cause CTLA-4 mRNA instability. CTLA-4 represents the related receptor of CD28 and binds also to CD80/CD86 on antigen-presenting cells (APC). Different mechanisms of action of CTLA-4 have been reported (Fernández-Mestre et al., 2009). One is the delivery of a negative signal to T cells, with rapid inhibition of T-cell activation. Another mechanism is B7 (CD80/CD86) sequestration on APC and induction of T-cell anergy (Carreno et al., 2000). In light of these findings, we can hypothesize that the delivery of negative signals via CTLA-4/CD80-CD86 interaction, which is required to limit T-cell activation/proliferation and regulate the autoimmune response is broken in PF patients resulting in the aggravation of disease symptoms. Our hypothesis is supported by recent data on the role of CTLA-4 therapy in AID. Indeed, an exogenous recombinant CTLA-4/Ig molecule infused to the patients ameliorated symptoms in several AIDs (Abrams et al., 1999; Riley & June, 2005).

In the current study and regarding the *ICOS* gene, an association between IVS1+173 *ICOS* gene polymorphism and Tunisian endemic PF was found. Interestingly, the carriers of the C allele revealed a relatively high risk for the development of pemphigus. Our result was in concordance with that of Narbutt et al. (2010) which found that thers10932029 C allele increases the risk of PV and PF suggesting a possible pathogenic role of the polymorphism in pemphigus pathogenesis. A recent study also revealed that this DNA sequence variation has an impact on the level of mRNA for both *CTLA4* and *ICOS* gene expression in T cells (Kaartinen, Lappalainen, Haimila, Autero, & Partanen, 2007). ICOS plays an important role in the maintenance of T-cell activation. The regulation of T-lymphocyte activation includes complex interaction between down regulatory properties of CTLA4 and the stimulatory action of CD28 and ICOS (Salomon & Bluestone, 2001). On the other hand, recent data showed that haplotypes in the *ICOS* gene correlate with IL-10 secretion, (Shilling et al., 2005) and this may be consistent with our results on the association between *ICOS* polymorphism and pemphigus development.

On the other hand, because of the strong linkage disequilibrium within a few haplotypes found in the chromosomal region on 2q33, it may be not easy to pinpoint the predisposing polymorphisms. Previously, Ueda et al. (2003) have postulated that the associations between 2q33 chromosome region polymorphisms with several AIDs are limited to the segment that includes the 3′ region of the *CTLA4* gene. Among these polymorphisms, the microsatellite $(AT)_n$ was the most associated marker. By the same, Cunninghame Graham, Wong,

McHugh, Whittaker, and Vyse (2006) found allelic variants located in the proximal and the distal 3′ flanking region of the *CTLA4*/*ICOS* promoter associated with systemic lupus erythematosus. In the present study, the same pattern of association was observed, indicating that susceptibility to PF is concentrated in the segment region between the *CTLA4* (AT) n repeats and *ICOS*+173TC.

Interestingly, discrepant results between endemic and sporadic patients can be explained by the different epidemiological features of the two groups, and confirm our previous ascertainment about the difference in their genetic background of susceptibility to the disease (Abida, Kallel-Sellami, et al., 2009; Ben Jmaa et al., 2017).

In conclusion, our study has independently replicated and confirmed a strong association of *CTLA4*(AT) repeat and *ICOS*>+173TC of 2q33.2 with the risk of PF in the Tunisian population, which suggests that there may be some common genetic factors shared by different ethnic populations. From a genetic point of view, we observed similar findings than the previous data in Brazilian endemic form. Understanding population similarities and differences in genetic susceptibility for a complex disease like PF will improve our understanding of the disease and clinical intervention.

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CONFLICT OF INTEREST

The authors disclose any conflict of interest.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to this study and approved the final version of the manuscript. Below is the contribution of each author to the manuscript: Abida Olfa performed the genetic and statistical analysis and drafted the manuscript. Ben Jmaa Mariem, Zouidi Ferjeni, and Fakhfakh Raouia contributed to data gathering and analysis. Bahloul Emna, Sellami Khadija, and Turki Hamida contributed to the clinical characterization of the disease and the clinical follow-up of patients. Mahfoudh Nadia contributed to the genotyping of the microsatellite marker. Masmoudi Hatem critically revised the manuscript for important intellectual content.

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