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RESEARCH

Variations in Suppressor Molecule *CTLA-4* Gene Are Related to Susceptibility to Multiple Myeloma in a Polish Population

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Abstract Various phenotype and functional T-cell abnormalities are observed in multiple myeloma (MM) patients. The aim of this study was to investigate the association between polymorphisms in the gene encoding cytotoxic T-lymphocyte antigen-4 (CTLA-4), a negative regulator of the T-lymphocyte immune response and susceptibility to multiple myeloma in a Polish population. Two hundred MM patients and 380 healthy subjects were genotyped for the following polymorphisms: *CTLA-4*c.49A>G, *CTLA-4*g.319C>T, *CTLA-4*g.*642AT(8_33), CT60 (*CTLA-4*g.*6230G>A), Jo31 (*CTLA-4*g.*10223G>T). Our study is the largest and most comprehensive evaluation to date of the association between genetic polymorphisms in the CTLA-4 molecule and multiple myeloma. It was found that *CTLA-4*c.49A>G[G], CT60[G], and Jo31[G] alleles were more frequently observed in MM patients than in controls (0.50 vs. 0.44, $p=0.03$, 0.65 vs. 0.58, $p=0.04$, and 0.63 vs. 0.57, $p=0.03$, respectively). Moreover, the haplotype *CTLA-4*c.49A>G[G], *CTLA-4*g.319C>T[C], *CTLA-4*g.*642AT(8_33) [8], CT60[G], Jo31[G] including all susceptibility alleles increases the risk of MM about fourfold (OR: 3.79, 95%CI: 2.08–6.89, $p=0.00001$). These findings indicate that genetic variations in the *CTLA-4* gene play role in susceptibility to multiple myeloma and warrant further investigation through replication studies.

Keywords CTLA-4 · Gene polymorphisms · MM

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Introduction

Multiple myeloma (MM) is a B-cell neoplasm characterized by the proliferation and accumulation of isotype-switched immunoglobulin-producing monoclonal plasma cells in the bone marrow and by overproduction of monoclonal immunoglobulins that can be detected in serum and/or urine. Previous studies reported that MM patients may exhibit a variety of T-cell abnormalities, such as a marked reduction in the proportions of CD4 and CD8 cells expressing co-stimulatory molecules, signal transduction components, and Th1/Th2 imbalance, particularly in advanced stages of MM [1–5]. The involvement of altered T-cell response in the development of murine plasmacytomas has also been reported [6]. The effective activation of naïve

T-cells requires two independent signals. The first, an antigen-specific signal, is sent via a unique antigen receptor, the T-cell receptor (TCR), on T-cells. The second signal, termed co-stimulation, is critical for allowing full activation, sustaining cell proliferation, preventing anergy and/or apoptosis, inducing differentiation to effector and memory status, and allowing cell-cell cooperation. CD28 is the primary T-cell co-stimulatory molecule and is constitutively expressed on the majority of T-cells. Cytotoxic T-cell antigen (CTLA-4) is a homologous molecule of CD28 which plays an inhibitory role in the early and late stages of T-cell activation [7]. CTLA-4 ligation provides a negative signal for regulation of the cell cycle and inhibits the activity of the transcriptional factors: nuclear factor-κB (NF-κB), nuclear factor of activated T-cells (NF-AT), and activator protein 1 (AP-1). Moreover, CTLA-4 binds to CD28 ligands (CD80 and CD86) with higher affinity and avidity and in that way also inhibits T-cell activation [8].

Several reports have indicated that *CTLA-4* gene polymorphisms are implicated in malignancies [9–14]. To the best of our knowledge, no large cohort-based study on gene polymorphisms of co-stimulatory and down-regulatory molecules has been performed in MM. Only one report was devoted to an association between the *CTLA-4*g.*642AT (8_33) polymorphism and susceptibility to MM [15]. Therefore we undertook a study to investigate the relationship between *CTLA-4*c.49A>G (rs231775), *CTLA-4*g.319C>T (rs5742909), *CTLA-4*g.*642AT(8_33), *CTLA-4*g6230G>A (CT60, rs3087243), *CTLA-4*g.10223G>T (Jo31, rs11571302) gene polymorphisms and MM incidence, age at disease onset, ISS, M component type, light chain, response to treatment, and time to disease progression.

The polymorphic markers we chose for our study are reported to be functional and to be associated with altered immune responses. The *CTLA-4*g.319C>T single nucleotide polymorphism (SNP) influences promoter activity and the expressions of both CTLA-4 mRNA in unstimulated cells and cell-surface CTLA-4 on activated cells [16–19]. The A>G transition at position 49 in exon (*CTLA-4c.49*A>G) causes a Thr/Ala substitution in the leader peptide [20]. This polymorphism affects the inhibitory function of CTLA-4 [16, 21]. The dinucleotide short repeat polymorphism *CTLA-4g.*642*AT(8_33) at position 642 in the 3′untranslated region (UTR) influences the level of mRNA transcription [22]. The *CTLA-4*g.*6230G>A (CT60) polymorphism was shown to be associated with variations in the mRNA level of soluble CTLA-4 [24]. Results published by us indicate that the Jo31 polymorphism together with CT60 is associated with the levels of membrane and cytoplasmic CTLA-4 in CD4+ T lymphocytes from multiple sclerosis patients [23] and with the variation of serum soluble CTLA-4 level in Graves-Basedow patients [24]. Moreover, all the investigated polymorphisms are associated with susceptibility to malignancies [9, 11–14].

The results from the present study indicate that *CTLA-4* gene polymorphisms are related to susceptibility to multiple myeloma in a Polish population.

Materials and Methods

Study Population

The study enrolled 200 unrelated Polish patients with multiple myeloma came from two centers: 148 from the Department of Haematology, Neoplastic Diseases & Bone Marrow Transplantation of the Medical University in Wroclaw, and 52 from the Department of Haematology of the State Hospital in Opole. There were 106 women and 94 men aged 33 to 85 (median±SD, 67±10.9 years), and the median age at diagnosis was 63.5±11.2 years. The diagnosis of multiple myeloma was based on criteria established by the International Myeloma Working Group [25]. Clinical stage was assessed according to the International Staging System (ISS) for Multiple Myeloma and was determined during enrolment in the study [26], i.e. stage 1 with serum β2microglobulin less than 3.5 mg/l +serum albumin ≥3.5 g/dl, stage 2 with neither stage 1 nor 3, and stage 3 with serum β2microglobulin level ≤5.5 mg/l. The patients were treated according to the melphalan+prednisone (MP); vincristine, doxorubicin (Adriamycin), dexamethasone (VAD); vincristine, melphalan, cyclophosphamide, prednisone (VMCP); or cyclophosphamide, thalidomide, dexamethasone (CTD) protocol. Table 1 summarizes the clinical characteristics of the MM patients.

The control group included 380 healthy Polish individuals originating from the same geographical area as the patients (214 female and 166 male) with the majority recruited from the blood bank in Wroclaw and others recruited among employees of the Institute of Immunology and Experimental Therapy. Genetic homogenity of the Polish population is observed, as reflected in virtually identical frequencies of H-Y polymorphisms in different regions of Poland [27]. All enrolled participants were informed about the study protocols and consent was obtained from each individual. Data on participation rate were not available. The study was approved by the local ethics committee.

Genotyping/Determination of Polymorphisms

A single nucleotide polymorphisms (SNPs) g.319C>T in the promoter region, c.49A>G in exon 1, and CT60 in the 3′UTR of the *CTLA-4* gene were examined by polymerase chain reaction–restriction fragment length polymorphism

Table 1 Patients' characteristics

Number of patients		200
Age (median, st. dev., range)		67±10.7 (33–87)
Age of onset (median, st. dev., range)		63.5±11.2 (32–84)
Gender (female/male)		106/94
International Staging System (ISS)	1	73
	2	66
	3	61
Ig subtype	IgG	115
	IgA	45
	IgM	5
	IgD	1
	Non-secretory	4
	Light chain	13
	others	17
Light chain type	κ	67%
	λ	33%
Number of controls		380
	volunteers of blood bank	329
	employee of Institute of Immunology& Experimental Therapy	51
Gender of controls (female/male)		214/166

(PCR-RFLP) using *Tru*I, *Bse*XI, and *Tail*I enzymes (Fermentas, Burlington Ontario, Canada). Table 2 lists the conditions of PCR product digestion with the restriction enzymes. Table 3 lists the primer sequences for PCR. The PCR reaction was carried out as described previously.

The Jo31 *CTLA-4* gene polymorphism was genotyped by the allelic discrimination method on the Applied Biosystems 7300 Real-Time PCR System using the TaqMan SNP Genotyping Assay-on -Demand (Applied Biosystems, Warrington, UK).

The *CTLA-4* 3′ UTR containing (AT)n repeats was amplified with a pair of primers (listed in Table 3) in which the 5′ ends of the forward primers were labelled with JOE and 6-FAM, respectively (Bionovo, Legnica, Poland). The detailed procedure is described in Suwalska et al. [9].

For quality controls we performed 5% to 10% re-typing with double blind check. For all SNPs additionally typing with other genotyping methods was used. Re-typing methods were as follows: g.319C>T SNP in the promoter region, c.49A>G in exon 1, and CT60 in the 3′ UTR of the *CTLA-4* gene were genotyped using PCR and then single-nucleotide primer-extension methods as described in Suwalska et al. [9], while the Jo31 SNP was genotyped using PCR and then single-nucleotide primer-extension methods as described in Karabon et al. [23].

The primers were designed according to the complete *CTLA-4* gene sequence derived from the NCBI Sequence Viewer.

Statistical Analyses

The evaluation of Hardy-Weinberg equilibrium was performed independently for the patients and control group by comparing the observed and expected frequencies of genotypes using χ^2 analysis. The χ^2 test was used to compare categorical data between patients with MM and controls. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated using the binary logistics regression model. The linkage disequilibrium (LD) coefficients $D'=D/D_{max}$ and r^2 for the pair of the most common alleles at each locus and haplotype frequencies for pairs of alleles were determined using the SHEsis program (http://202.120.7.14/analysis/myAnalysis.php) [28]. Haplotypes with frequencies lower than 0.03 were not considered.

Results

CTLA-4 Polymorphisms and Susceptibility to MM

The distributions of the alleles and genotypes of all studied polymorphisms in the MM patients and the healthy control group are shown in Table 4. Neither in cases nor in controls was deviation from Hardy-Weinberg equilibrium observed

Table 2 Conditions for PCR product digestion with restriction enzymes

Amplicon [bp]	SNP	Enzyme	Temperature and duration of digestion	Digestion products visible on gel [bp]
814	*CTLA-4*g.319C>T	*Tru*I	65°C, 3 h	C: 51, 101, 100, 562 T: 51, 101, 100, 94, 468
814	*CTLA-4*c.49A>G	*Bse*XI	65°C, 4 h	A: 207, 607 G: 207, 508, 99
806	CT60	*Tail*I	65°C, 4 h	G: 419, 236, 151 A: 236, 570

Table 3 Primer sequences and annealing temperatures used for *CD28*, *CTLA-4*, and *ICOS* genotyping.

Polymorphic site	Type of reaction	Primer sequence	Product size [bp]	T_m [°C]
*CTLA-4*c.49A>G (rs231775) *CTLA-4*g.319C>T (rs5742909)	PCR	F: 5′ –TCT TTT CCg CCT ATT TTC AgT T– 3′ R: 5′ – CAC CTC CTC CAT CTT CAT gCT CC – 3′	814	58
CT60 (rs3087243)	PCR	F: 5′ – Tgg gCC CAA TTC TTA CAA AC – 3′ R: 5′ – CAg gAT gTg gAg gTC AAA AA– 3′	806	58
*CTLA-4*g.*642AT(8_33)	PCR	F: (JOE) 5′–gCC AgT gAT gCT AAA ggT Tg–3′ R: 5′–TgC CAg TTC CCT ACA AgA Ag–3′	82–132	52

(Table 4). The odds ratios (OD) and 95% confidence intervals (CI) in Table 4 are shown for the codominant model. For the microsatellite polymorphism *CTLA-4g.***642*AT(8_33) the alleles were grouped arbitrarily into two groups, i.e. short ([8] 8 repeats) and long ([>8], >8 repeats) alleles.

The exon 1 *CTLA-4* gene polymorphism at position +49 was found to be associated with MM. The frequency of the *CTLA-4*c.49A>G[G] allele was significantly higher in the MM patients than in the controls (p=0.03), with an estimated odds ratio (OR) of 1.31 and a 95% confidence interval (CI) of 1.03–1.68. Moreover, the distributions of the genotypes were different in the patients and controls. Individuals possessing [G] alleles (genotypes [GG] and [AG]) were present significantly more frequently among patients than controls (p=0.02, OR: 1.61, 95%CI: 1.08–2.44).

Moreover, the Jo31 SNP contributed to the risk of MM. The Jo31 [G] allele and [GG] genotype were associated with increased the risk of MM (OR: 1.32, p=0.03, 95%CI: 1.03–1.69 and OR: 1.46, p=0.04, 95%CI: 1.02–2.08, respectively) (Table 4).

Additionally we noted a significantly higher frequency of the CT60 [G] allele and a trend toward over-expressing the [GG] genotype in MM patients compared with controls (p=0.04, OR: 1.3, 95%CI: 1.11–1.68 and p=0.07, OR: 1.41, 95%CI: 0.97–1.99, respectively) (Table 4).

The distribution of alleles and genotypes in *CTLA-4*g.319C>T and *CTLA-4*g.*642AT(8_33), polymorphisms did not differ between the group of MM patients and controls.

The global distributions of the haplotypes differed significantly between the cases and controls ($p_{\text{after Bonferroni correction}}$=7.5e-005) (Table 5). The haplotype *CTLA-4*c.49A>G[G]/*CTLA-4*g.319C>T[C]/*CTLA-4*g.*642AT(8_33)[8]/CT60[G]/Jo31[G] was overrepresented in the cases compared with the controls (OR: 3.79, 95%CI: 2.08–6.89, p=0.00001).

CTLA-4 Gene Polymorphisms and Clinical Data in Multiple Myeloma Patients

Because multiple myeloma occurs more frequently in males than in females, we conducted a separate association test of all the studied polymorphic markers, i.e., *CTLA-4*c.49A>G, *CTLA-4*g.319C>T, *CTLA-4*g.*642AT(8_33), CT60, and Jo31 in the *CTLA-4* gene in male and female participants. We noted an even stronger association between the *CTLA-4*c.49A>G[G] allele and susceptibility to MM in males (OR:1.61, 95%CI:1.11–2.33, p=0.01). None of the studied polymorphic markers correlated with the clinical data regarding: age at disease onset, ISS, M component type, light chain, response to treatment, or time to disease progression.

Linkage Disequilibrium

The investigated polymorphisms in the 3′ UTR, namely, Jo31, CT60, and *CTLA-4*g.*642AT(8_33) were in strong linkage disequilibrium with each other expressed by either D′ or r^2 (Table 6). In the case of pairs *CTLA-4*c.49A>G and *CTLA-4*g.319C>T; *CTLA-4*c.49A>G and *CTLA-4*g.*642AT(8_33); *CTLA-4*g.319C>T and *CTLA-4*g.*642AT(8_33); *CTLA-4*c.49A>G and CT60; *CTLA-4*c.49A>G and Jo31; *CTLA-4*g.319C>T and CT60; and *CTLA-4*g.319C>T and Jo31 linkage disequilibrium between them was expressed by D′ (Table 6).

Discussion

Both genetic and environmental factors have been associated with an increased risk of MM.

Family history of MM in a first-degree relative suggested that genetic variation plays a role in the etiology of multiple myeloma [29]. The genetic susceptibility to multiple myeloma has not been extensively studied, although some studies have evaluated polymorphisms in immune response genes especially cytokine [30–32]. Others were devoted to variations in genes involved in xenobiotics metabolism [33], DNA repair [34], the cell cycle and apoptosis [35].

Genes encoding proteins involved in T-cell activation and suppression have been considered as candidate genes for many autoimmune [36–38] and, recently, also for neoplastic diseases [9, 13, 14]. Only one study was devoted to the association of one polymorphic marker of the *CTLA-4* gene and multiple myeloma [15].

Table 4 *CTLA-4c.49A>G*, *CTLA-4g.319C>T*, *CTLA-4g.*642AT(8_32)*, *CT60G>A*, genotypes and alleles frequencies in MM patients and controls

			Multiple myeloma n=200	Controls group n=380	p	OR	95%CI	Global p
CTLA-4c.49A>G	Allele	A	199 (50.0%)	417 (56.3%)	0.03	0.77	0.60–0.97	
		G	199 (50.0%)	318 (43.7%)		1.31	1.03–1.68	
	Genotype	A/A	48 (24.1%)	124 (33.4%)	reference			
		A/G	103 (51.8%)	169 (45.6%)	0.03	1.57	1.04–2.38	0.07
		G/G	48 (24.1%)	75 (21.0%)	0.04	1.65	1.01–1.86	
	Carriers of	G+	151 (76.9%)	244 (66.6%)	0.02	1.61	1.08–2.44	
		G-	48 (24.1%)	124 (33.4%)		0.63	0.41–0.93	
CTLA-4g.319C>T	Allele	C	356 (89.4%)	672 (90.2%)	0.64	0.90	0.61–1.36	
		T	42 (10.6%)	72 (9.8%)		1.10	0.73–1.64	
	Genotype	CC	155 (79.5%)	297 (80.9%)	reference			
		CT	40 (20.5%)	68 (18.5%)	0.59	1.13	0.73–1.74	0.43
		TT	0 (0.0%)	2 (0.6%)	0.86	–	–	
	Carriers of	T+	40 (20.5%)	70 (19.1%)	0.68	1.09	0.71–1.698	
		T-	155 (79.5%)	297 (80.9%)		0.91	0.59–1.41	
*CTLA-4g.*642AT(8_32)*	Allele	8	190 (47.5%)	350 (46.1%)	0.64	1.06	0.83–1.35	
		>8	210 (52.5%)	410 (53.9%)		0.94	0.74–1.20	
	Genotype	8/8	55 (27.5%)	94 (24.7%)	reference			
		8/>8	80 (40.0%)	162 (42.6%)	0.43	0.84	0.55–1.82	0.74
		>8/>8	65 (32.5%)	124 (32.6%)	0.63	0.89	0.57–1.40	
	Carriers of	8+	135 (67.5%)	256 (68.4%)	0.97	1.00	0.70–1.45	
		8−	65 (32.5%)	124 (32.6%)		0.99	0.69–1.43	
CT60G>A	Allele	G	249 (64.5%)	444 (58.4%)	0.04	1.30	1.11–1.68	
		A	137 (35.5%)	316 (41.76%)		0.77	0.60–0.90	
	Genotype	G G	81 (41.7%)	128 (34.2%)	reference			
		G A	88 (45.8%)	180 (48.1%)	0.18	0.93	0.64–1.28	0.14
		A A	24 (12.5%)	66 (17.6%)	0.04	0.57	0.33–99	
	Carriers of	A+	112 (58.3%)	246 (65.8%)	0.07	0.71	0.50–1.03	
		A-	81 (41.7%)	128 (34.2%)		1.40	0.97–1.99	
Jo31G>T	Allele	G	253 (63.2%)	425 (56.5%)	0.03	1.32	1.03–1.69	
		T	147 (36.8%)	327 (43.5%)		0.76	0.59–0.97	
	Genotype	G G	80 (40.0%)	118 (31.4%)	reference			
		G T	93 (46.5%)	189 (50.3%)	0.39	0.86	0.61–1.21	0.08
		T T	27 (13.5%)	69 (18.4%)	0.14	0.69	0.43–1.23	
	Carriers of	A+	120 (60.0%)	258 (68.6%)	0.04	0.68	0.48–0.98	
		A-	80 (40.0%)	118 (31.4%)		1.46	1.02–2.08	

We found that of the five polymorphisms studied in the *CTLA-4* gene, three of them (*CTLA-4*c.49A>G, CT60, and Jo31) were significantly associated with MM. We observed that *CTLA-4*c.49A>G[G] allele, the Jo31[G] allele and [GG] genotype and the CT60[G] allele increased the risk of developing MM.

It is of great interest that genetic variants of the studied polymorphisms have been previously reported to contribute to a lower expression level of mRNA and/or CTLA-4 molecule expression on T-cells [16, 21, 23, 39]. *CTLA-4*c.49A>G transition is a functional polymorphism which influences T-cell activation and therefore might play a role in altered T-cell regulation. Presence of [GG] genotype was shown to be associated with significantly higher activation of T lymphocytes and higher proliferation than [AA] genotype. The protein product coded by *CTLA-4*c.49A>G [GG] genotype CTLA-4^{17}Ala had lower capacity to bind B7.1 and a weaker inhibitory effect on T-cell activation compared with CTLA-4^{17}Thr [40]. It has also been postulated that the *CTLA-4*c.49A>G polymorphism in the leader sequence may influence rates of endocytosis or surface trafficking [21], the glycosylation of CTLA-4, and

Table 5 Haplotype estimation analysis of investigated polymorphisms within *CTLA-4* gene

*CTLA-4*c.49A>G/ *CTLA-4*g.319C>T/ *CTLA-4*g.*642AT (8-32)/ CT60 G>A/ Jo31 G>T	Multiple myeloma *n*=200	Controls group *n*=380	OR	95%CI	p	p after Bonferroni correction
A C 8 A T	97.72 (0.28)	256.28 (0.36)	0.70	[0.53~0.94]	0.02	0.1
A C >8 G G	22.61 (0.07)	36.35 (0.05)	1.33	[0.78~2.29]	0.30	
A T >8 G G	24.02 (0.07)	50.04 (0.07)	1.01	[0.61~1.68]	0.97	
G C 8 A T	16.54 (0.05)	17.21 (0.03)	2.08	[1.04~4.14]	0.03	0.15
G C 8 G G	30.33 (0.09)	18.17 (0.03)	3.79	[2.08~6.89]	3.67e-006	1.0e-005
G C >8 G G	111.33 (0.32)	257.64 (0.37)	0.854	[0.64~1.13]	0.27	

Global result:

Total control=700.0, total case=346.0, Global chi2 is 29.923019 while df=5, Fisher's p value is 1.58e-005, $p_{\text{after Bonferroni correction}}$=7.5e-005

intracellular/surface partitioning, and in that way alter inhibitory function of that molecule [16]. Our previous results indicated that presence of G alleles in polymorphic sites CT60 and Jo31 was associated with the lower levels of membrane and cytoplasmic CTLA-4 in CD4+ T lymphocytes from multiple sclerosis patients [23] and with the variation of the serum soluble CTLA-4 level in Graves-Graves' disease patients [24].

In fact a marked reduction, particular in advanced stage of MM, in the proportion of CD4 and CD8 cells expressing CTLA-4, CD28, CD3zeta, p56lck, ZAP-70 and PI3-k, what indicate that profound and multiple T-cell signalling defects, from the surface and down-stream, consistent with involvement of a master T-cell function, especially in advanced stage MM [1].

In the only report about an association of *CTLA-4* gene polymorphism and MM published previously by Zheng et al. [15], the prevalence of longer *CTLA-4*g.*642AT(8_33) alleles, which are associated with lower stability of mRNA for CTLA-4 was described. In our study we also observed nonsignificantly increased frequency of longer alleles.

Our findings regarding *CTLA-4* gene polymorphisms and susceptibility to MM are different from those we obtained for B-cell chronic lymphocytic leukaemia (B-CLL) [9], in which the *CTLA-4*g.319C>T[T] allele was significantly associated with disease. Although it seems surprising, population studies based on a positive family history of lymphoproliferative neoplasms indicated that the genetic pathway involved in the etiology of chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), and Hodgkin's lymphoma (HL) is different from that of MM. It was shown that first-degree relatives of chronic CLL patients are at higher risk of developing CLL, NHL, and HL, but not at increased risk of developing MM [41, 42]. In contrast, first-degree relatives of MM patients are not at risk of developing B-CLL, CLL, NHL, or HL [17].

A limitation of this study is the relatively small group of patients, but it should be mentioned that the population risk of MM is approximately 0.00003 and it is difficult to collect a bigger homogeneous ethnically matched cohort. Therefore our findings should be confirmed by an independent study on a larger cohort.

Our original findings of the prevalence of the *CTLA-4*c.49A>G[G], Jo31[G], and CT60[G] alleles suggested that genetic variations in the *CTLA-4* gene play a role in multiple myeloma and warrant further investigation through replication studies.

Table 6 Linkage disequilibrium analysis in *CTLA-4* gene

	*CTLA-4*c.49A>G		*CTLA-4*g.*642AT(8_33)		CT60		Jo31	
	D′	r^2	D′	r^2	D′	r^2	D′	r^2
*CTLA-4*g.319C>T	0.883	0.074	0.546	0.028	0.906	0.060	0.805	0.050
*CTLA-4*c.49A>G			0.567	0.237	0.769	0.320	0.770	0.353
*CTLA-4*g.*642AT(8_33)					0.924	0.651	0.818	0.537
CT60							0.901	0.752

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Conflict of interests The authors declare that they have no competing interests.

Contributions to the study LK designed of the study, genotyped and drafted the manuscript (MS); EP, AJ, AT, AJ analyses and interpretation; MK, SP, DW clinical survey, KK revised the MS, IF was the guarantor. All authors approved the manuscript.

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