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ORIGINAL ARTICLE

Is the Genetic Background of Co-Stimulatory CD28/CTLA-4 Pathway the Risk Factor for Prostate Cancer?

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Abstract The impairment of immunological surveillance caused by aberrant T cell activation can lead to an inadequate anti-tumor response. Therefore, deregulation in co-stimulatory pathway might be associated with cancer susceptibility. Here we undertook a prospective study to investigate whether genetic variations in gene encoding molecule CD28 and CTLA-4 playing pivotal role in regulating adoptive immune response can influence susceptibility to prostate cancer. Single nucleotide polymorphisms (SNPs) in *CTLA-4* and *CD28* genes were genotyped in 301 prostate cancer (PCa) patients and 301 controls. The distributions of the genotypes and haplotypes in the *CTLA-4/CD28* SNPs were similar in both studied groups. However, the overrepresentation of carriers of *CTLA-4*c.49A>G[A] allele and carriers of *CTLA-4*g.319C>T[T] allele in PCa as compared to controls was observed (p = 0.082 and p = 0.13, respectively). The risk of disease was higher (OR 1.78) for carriers of both susceptibility alleles as compared to carriers of protective genotypes (p = 0.03). The *CTLA-4*c.49A>G and *CTLA-4*g.319C>T SNPs might be considered as low risk susceptibility *locus* for PCa.

Keywords CTLA-4 · CD28 · Gene polymorphisms · Prostate cancer

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Introduction

Prostate cancer is a common malignancy among men in Western and Asian Countries and one of the most frequent causes of mortality and morbidity [1]. The mechanism(s) underlying the development of prostate cancer are still unknown, but it is postulated that both environmental and genetic factors play role [2, 3].

Numerous epidemiological studies such as case-control, cohort and genome-wide association studies have shown the role of low-risk genetic variation in susceptibility to PCa [4]. Genome-wide association studies [5–9] have identified 77 susceptibility *loci* for prostate cancer that explain approximately 30% of the familial risk of prostate cancer. To date, the number of genes identified as predisposing to PCa is limited and includes, among others, genes involved in the DNA damage response pathway, in cell-cycle regulation, signal transductions, angiogenesis and the most interesting for us – immune response against tumor.

One of the most important mechanisms in cancer development and progression is the impairment of immunological surveillance caused by aberrant T cell activation, which leads to an inadequate anti-tumor response [10]. The level of T cell activation depends on the balance between co-stimulatory and co-inhibitory signals delivered by co-signaling molecules. CD28, constitutively expressed on the majority of T cells, is the primary T-cell co-stimulatory molecule which enhances T-cell activation and proliferation [11]. CTLA-4 is a down-regulatory molecule only minimally expressed on resting T cells, and it is transiently up-regulated after stimulation [11].

CD28 and CTLA-4 are structurally similar and they share 30% of sequence homology [12]. Both CD28 and CTLA-4 bind to the ligands: CD80 and CD86 via MYPPY motif, but due to the binding of one CTLA-4 homodimer to two CD80 molecules the avidity of this molecule is higher than for CD28

[13]. As was shown on animal model the absence of CTLA-4 signaling causes constitutive activation of protein tyrosine kinases: FYN, LCK and ZAP-70 [14]. CTLA-4 has YVKM motif which binds the SHP2 domain of tyrosine phosphatase SHP2, what results in defosforylation of CD3ζ chain and reducing the signaling potential of T-cell receptor. Furthermore, the phosphatase PP2A binds to lysine rich motif on the cytoplasmic tail of CTLA-4, what results in decreased downstream AKT phosphorylation [15].

The abnormal CTLA-4 antigen functioning may be one of the mechanisms responsible for tumor development. Blocking CTLA-4 antigen on the surface of T cells promotes the regression of experimentally induced tumors in mice, suggesting the involvement of this receptor in the pathogenesis of neoplasms [16, 17]. Encouraging results obtained in animal models were the basis for the application of a CTLA-4 blockade in the treatment of different cancers in humans [18]. The exact mechanism of antitumor response caused by blocking CTLA-4 is not well established, but it is postulated that it may be a combination of several effects. CTLA-4 blocking might act by lowering the threshold needed for T cell activation, by reduction in the number of Tregs and also by reducing the release of suppressive molecules such as: IL-10, IL-35, TGF-β as well as idoleamine 2,3 dioxygenase (IDO). These mechanisms enhance an antitumor immunity by promoting T-cell activation and cytotixic T lymphocyte proliferation (reviewed in [19]).

The prostate cancer was one of the first cancers where this innovative approach was examined. Currently, in clinical trials, two IgG monoclonal antibodies, ipilimumab and tremelimumab [20], are being tested mainly as part of complex immunomodulatory maneuvers. However, anti-CTLA-4 treatment may cause immune related adverse effects (irAE), such as: skin lesions (rush, pruritus and vitiligo), colitis, and less frequently hepatitis, hypophysitis, thyroiditis and some rare events: sarcoidosis, uveitis, polymyalgia. On the other hand, the occurrence of irAEs is positively related to oncologic response. It was shown that 60% of patients treated with CTLA-4 blocking presenting with irAEs experienced clinical response (partial or complete) or at least cancer stabilization [21].

Immune response against prostate tumor is evidenced by intratumoral leukocyte infiltration and inflammatory pathway activation [22, 23], which suggests that host immune system can mount a natural antitumor response that employs both initiate and adoptive branches. Therefore, we undertook a prospective study to investigate, whether genetic variations in genes encoding molecule CD28 and CTLA-4 playing pivotal role in regulating adoptive immune response, can influence susceptibility to prostate cancer. We have focused our attention on single nucleotide polymorphisms (SNPs) in the *CTLA-4* gene: *CTLA-4*c.49A>G (rs231775), *CTLA-4*g.319C>T (rs5742909), *CTLA-4*g.*6230G>A (CT60) (rs3087243), *CTLA-4*g.*10223G>T (Jo31) (rs11571302) and *CD28* gene: *CD28*c.17+3T>C (rs3116496), *CD28*c.-1042G>A (rs3181098).

To the best of our knowledge it is the first study on this subject.

Materials and Methods

We prospectively recruited 301 prostate tumor patients treated at the Department of Urology and Oncologic Urology, Wroclaw Medical University, between March 2010 and March 2014. All patients were diagnosed with prostate cancer on prostate biopsy before and were admitted for curative treatment (prostatectomy or radiotherapy) to the ward. After giving written informed consent all subjects were enrolled to the study before treatment initiation. The control group consisted of 301 healthy men originating from the same geographical area as the patients, recruited from the blood bank in Wroclaw or from employees of the Institute of Immunology and Experimental Therapy.

The study was approved by the local ethics committee, and all participants gave written informed consent.

Genotyping

The SNPs g.319C>T in the promoter region, c.49A>G in exon 1, and CT60 in the 3′ untranslated region (UTR) of the *CTLA-4* gene were examined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) using *Tru*I, *Bse*XI, and *Tail*I enzymes (Fermentas, Burlington Ontario, Canada), as described previously [24].

The Jo31, *CD28c*.17+3 T>C and *CD28*c.-1042G>A SNPs were genotyped using the TaqMan®SNP Genotyping Assays: C__2415786_10, C__25922478_10_and C__27467172_10, respectively (Applied Biosystems, Foster City, USA).

For quality controls, we performed 10% re-typing with a double-blind check. Additionally, we conducted retyping using the following method: the SNPs g.319C>T, c.49A>G, and CT60 in the *CTLA-4* gene were typed using TaqMan®SNP Genotyping Assays, while the Jo31 and *CD28*c.17+3T>C SNPs were identified using PCR followed by single-nucleotide primer-extension methods as described in Suwalska et al. [12].

Statistical Analyses

Hardy-Weinberg equilibrium (HWE) was evaluated independently for the patients and the controls by comparing the observed and expected frequencies of genotypes using χ^2 analysis. The χ^2 test was used to compare categorical data between groups. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using the binary logistic regression model.

The haplotype frequencies for pairs of alleles were determined using the SHEsis program [25]. Haplotypes with frequencies lower than 0.03 were not included in further analyses. The significance of two genetic factors affecting the risk of PCa was determined with the use of Svejgaard and Ryder method [26]. Differences were considered statistically significant if $p < 0.05$.

Results

Each polymorphism in the *CTLA-4* and *CD28* genes was in HWE in both the cases and the controls.

CTLA-4 and *CD28* Gene Polymorphisms and Susceptibility to the Development of Prostate Cancer

The distributions of the alleles and genotypes for all tested polymorphisms are presented in Table 1. The analysis revealed that the frequency of alleles, and genotypes for the investigated polymorphisms in the *CTLA-4* and *CD28* genes did not significantly differ between the PCa patients and the controls. However, we observed an overrepresentation of individuals possessing the *CTLA-4*c.49A>G[A] allele compared to the *CTLA-4*c.49A>G[GG] genotype among the PCa patients (0.86 vs 0.80, $p = 0.082$, OR 1.46, 95% CI: 0.95–2.25). There was also not significant prevalence of the carriers of [T] allele in *CTLA-4*g.319C>T SNP in PCa patients (0.26 vs 0.20, $p = 0.13$, OR 1.35, 95% CI: 0.92–1.97).

To assess the significance of two genetic factors associated with PCa risk the Svejgaard and Ryder [26] method was employed. The following factors were considered: factor A – possessing of [A] allele at *CTLA-4*c.49A>G (genotype [AA] and [AG]); factor B - possessing of [T] allele at *CTLA-4*g.319C>T (genotype [CT] and [TT]). The results of that analysis are presented in Table 2. The frequency of the carriers of susceptibility alleles for both SNPs (factor A and factor B) was significantly higher in the PCa patients as compared to the individuals lacking factor A and B (test [8] Table 2) and possessing of both factors A and B increased the risk of disease 1.78 fold (OR 1.78, 95% CI: 1.06–3.01, $p = 0.03$). In addition, we observed the tendency to higher risk of PCa in the individuals possessing factor A and lacking factor B as compared to the group of factors A and B negative persons (test [4]) (OR 1.51, 95% CI: 0.96–2.37, $p = 0.07$).

Since *CD28* and *CTLA-4* genes are located next to each other on the same chromosome we have performed haplotype estimation analysis (Table 3). There were no differences in haplotype distribution between the PCa patients and the controls (Global $p = 0.85$). The most common haplotype for both groups was the haplotype consisted of wild alleles *CTLA-4*c.49A>G[G]/*CTLA-4*g.319C>T[C]/CT60[G]/Jo31[G]/*CD28*c.17+3T>C[T]/*CD28*c.-1042G>A[G] (0.26 for PCa and 0.28 for controls).

Discussion

PCa is complex malignancy associated with multiple factors: age, gene and environment. Host immune system can mount a natural antitumor response that employs both initiate and adoptive branches, what was evidenced by intratumoral leukocyte infiltration and inflammatory pathway activation in prostate tumors [22, 23].

The polymorphisms in co-stimulatory molecule genes were shown to be genetic susceptibility factors for several human cancers [27–32], therefore we hypothesized that they might influence susceptibility to prostate cancer in Polish population.

Firstly, *CTLA-4* genetic variability was investigated in different ethnic groups in autoimmune diseases, but the results of the study were conflicting. Recent series of meta-analysis pointed out, that *CTLA-4 locus* is rather general autoimmune than disease-specific genetic risk factor, especially the exon 1 and 3'UTR located markers, while their association with diseases susceptibility depends on the ethnicity. For example, it was shown that *CTLA-4*c.49A>G[G] and CT60[G] alleles are risk factors, especially in Caucasians or Asians, for: autoimmune thyroid diseases [33], autoimmune adrenal insufficiency [34], type 1 diabetes [35], rheumatoid arthritis [36] and systemic lupus erythematosus [37, 38]. The only association between *CTLA-4*g.319C>T and autoimmune disease susceptibility was observed for SLE in Asians [38].

Nowadays it is well established that polymorphisms in *CTLA-4* gene also confer susceptibility to cancers. In the present work we found the not statistically significant predominance of carriers of *CTLA-4*c.49A>G[A] allele in PCa patients as compared to controls. The A>G transition at position 49 in exon 1 (*CTLA-4*c.49A>G) causes a Thr/Ala substitution in the leader peptide and affects the inhibitory function of the CTLA-4 [39–41], therefore, might play a role in antitumor immunity. The presence of [AA] genotype was shown to be associated with significantly lower activation and proliferation of T lymphocytes than [GG] genotype. The protein product coded by *CTLA-4*c.49A>G[AA] genotype CTLA-4^{17}Thr had higher capacity to bind B7.1 and a stronger inhibitory effect on T-cell activation compared with CTLA-4^{17}Ala [41]. 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 [39], the glycosylation of CTLA-4, and intracellular/surface partitioning, and in that way alter inhibitory function of that molecule [40]. The role of the *CTLA-4*c.49*A>G*[A] allele as a risk factor for cancer development was shown in many types of cancers, such as esophageal cancer, gastric cardia cancer [41], non-Hodgkin's lymphoma [28], breast cancer [27, 41], and renal cancer [42].

Interestingly, the opposite results for this SNP were shown for mucosa-associated lymphoid tissue lymphoma [43] and

Table 1 Distribution of alleles and genotypes of the following SNPs: *CTLA-4*c.49A>G, *CTLA-4*g.319C>T, *CTLA-4*g.*6230G>A (CT60), *CTLA-4*g.*10223G>T (Jo31), *CD28*c.17+3T>C and *CD28*c.-1042G>A in prostate cancer patients compared with controls

SNP			Prostate cancer $N = 301$	Controls men $N = 301$	OR (95%CI)	p
CTLA-4	Genotype	A A	104 (34.6)	100 (33.2)	Ref	-
c.49A>G		A G	154 (51.2)	142 (47.2)	1.04 (0.73–1.49)	0.82
(rs231775)		G G	43 (14.3)	59 (19.6)	0.70 (0.43–1.13)	0.15
	$Chi^2 = 3.07$, df = 2, $p = 0.22$					
	HWE:					
	Cases: $Chi^2 = 1.35$, $p = 0.24$			Controls: $Chi^2 = 0.45$, $p = 0.50$		
	Carriers	A+	258 (85.5)	242 (80.4)	1.46 (0.95–2.25)	0.082
		G G	43 (14.3)	59 (19.6)	0.68 (0.44–1.05)	
CTLA-4	Genotype	C C	223 (74.1)	239 (79.4)	Ref	-
g.319C>T		C T	76 (25.2)	60 (19.9)	1.36 (0.92–1.99)	0.12
(rs5742909)		T T	2 (0.7)	2 (0.7)	1.07 (0.15–7.67)	0.95
	$Chi^2 = 2.44$, df = 2, $p = 0.30$					
	Carriers	T+	78 (25.9)	62 (20.6)	1.35 (0.92–1.97)	0.12
		C C	223 (74.1)	239 (79.4)	0.74 (0.51–1.08)	
CT60	Genotype	G G	113 (37.5)	109 (36.2)	Ref	-
(rs3087243)		A G	141 (46.8)	150 (49.8)	0.91 (0.64–1.29)	0.58
		A A	47 (16.5)	42 (14.0)	1.08 (0.66–1.77)	0.76
	$Chi^2 = 0.63$, df = 2, $p = 0.73$					
	HWE:					
	Cases: $Chi^2 = 0.07$, $p = 0.78$			Controls: $Chi^2 = 0.71$, $p = 0.40$		
	Carriers	G G	113 (37.5)	109 (36.2)	1.06 (0.76–1.47)	0.74
		A+	188 (62.5)	192 (63.8)	0.94 (0.68–1.32)	
Jo31	Genotype	G G	100 (33.2)	105 (34.9)	Ref.	-
(rs11571302)		G T	148 (49.2)	147 (48.8)	1.06 (0.74–1.51)	0.76
		T T	53 (17.6)	49 (16.3)	1.14 (0.71–1.83)	0.60
	$Chi^2 = 0.28$, df = 2 $p = 0.87$					
	HWE:					
	Cases: $Chi^2 = 0.02$, $p = 0.89$			Controls: $Chi^2 = 0.04$, $p = 0.83$		
	Carriers	G G	100 (33.2)	105 (34.9)	0.93 (0.66–1.30)	0.67
		T+	201 (66.8)	196 (65.1)	1.08 (0.77–1.51)	
CD28	Genotype	T T	207 (68.8)	211 (70.1)	Ref.	-
c.17+3T>C (rs3116496)		C T	86 (28.6)	84 (27.9)	1.04 (0.73–1.49)	0.81
		C C	8 (2.7)	6 (2.0)	1.36 (0.46–3.99)	0.58
	$Chi^2 = 0.35$, df = 2, $p = 0.84$					
	HWE:					
	Cases: $Chi^2 = 0.07$, $p = 0.79$			Controls: $Chi^2 = 0.07$, $p = 0.79$		
	Carriers	T T	207 (68.8)	211 (70.1)	0.94 (0.66–1.33)	0.72
		C+	94 (31.2)	90 (29.9)	1.06 (0.75–1.51)	
CD28	Genotype	G G	152 (50.5)	139 (46.2)	Ref.	-
c.-1042G>A (rs3181098)		A G	121 (40.2)	133 (44.2)	0.83 (0.59–1.17)	0.28
		A A	28 (9.3)	29 (9.6)	0.88 (0.50–1.56)	0.67
	$Chi^2 = 1.16$, df = 2, $p = 0.56$					
	HWE					
	Cases: $Chi^2 = 0.30$, $p = 0.58$			Controls: $Chi^2 = 0.12$, $p = 0.73$		
	Carriers	G G	152 (50.5)	139 (46.2)	1.19 (0.86–1.64)	0.30
		A+	149 (49.5)	162 (53.8)	0.84 (0.61–1.16)	

multiple myeloma [44], while no associations with cancer risk were observed for colorectal cancer [45], chronic lymphocytic leukemia [30], cervical squamous cell carcinoma [29], malignant melanoma [46], or non-malignant melanoma [47].

Resuming, in our study we observed the trend for overrepresentation of carriers of [A] allele among PCa patients. Although in the present literature this is the first study on an association between the *CTLA-4* gene polymorphisms and

Table 2 Analysis of the associations between two genetic factors carriers of *CTLA-4*c.49A>G[A] allele and *CTLA-4* g.319C>T[T] allele with prostate cancer risk using Svejgaard and Ryder method [26]

Basic data	Factor A: *CTLA-4*c.49A>G (rs231775) [AA] + [AG] Factor B: *CTLA-4*g.319C>T (rs5742909) [CT] + [TT]				
	PCa patients *n* = 301		Control group *n* = 300		
A+, B+	75		62		
A+, B-	183		179		
A-, B+	3		0		
A-, B-	40		59		
Test	OR	*p*	95%CI	Comparison	Individual association
[1] A	1.46	0.08	0.95–2.25		
[2] B	1.35	0.13	0.92–1.97		
[3] ++ vs − +	NA	0.33*	NA	A in B-positive	A association
[4] + − vs --	1.51	0.07	0.96–2.37	A in B-negative	
[5] ++ vs + −	1.18	0.40	0.80–1.76	B in A-positive	B association
[6] − + vs --	NA	0.14*	NA	B in A-negative	
[7] + − vs − +	NA	0.26*	NA	Differences between A and B association	
[8] ++ vs --	1.78	0.03	1.06–3.01	Combined association	

*- *p*-value after Yate's correction (when at least one $n \leq 5$)

prostate cancer, but *CTLA-4*c.49A>G[A] allele is established as a risk factor for several types of cancers, for example: cervical cancer [48], lung cancer and breast cancer in European [49].

Similarly for the promoter *CTLA-4*g.319C>T polymorphism we have noticed the prevalence of carriers of [T] allele in PCa patients as compared to healthy man. The *CTLA-4*g.319C>T SNP influences promoter activity and the expressions of both CTLA-4 mRNA in unstimulated cells and cell-surface CTLA-4 on activated cells [50, 51]. Therefore the [T] allele may contribute to the upregulation of the expression of the down-regulatory CTLA-4 molecule, inhibition of the activation of T lymphocytes, and eventually limitation of the potency of antitumor immunity, and in that way susceptibility to cancer. Previously we have shown an association between this promoter polymorphism and B-cell chronic lymphocytic leukemia [30]. Moreover, it was found by us and others that the same allele confers susceptibility to female-related cancers: sporadic breast cancer [31, 48], and cervical cancer [29, 32] as well as non-small cell lung cancer in women [24]. In contrast to our results *CTLA-4*g.319C>T polymorphism was not associated with lung cancer (without stratification by gender) [41] or other cancers, such as colon cancer [42], colorectal cancer [52] or multiple myeloma [44]. The meta-analysis performed by Zhang et al. [49] indicated this polymorphism as related to cancer risk in the Europeans.

Taking together, the individuals possessing two susceptibility alleles for both: *CTLA-4*c.49A>G and *CTLA-4*g.319C>T SNPs have higher risk of development PCa as compared to individuals with two protective genotypes.

The functional role of *CTLA-4*g.*6230G>A (CT60) polymorphism has been shown by Ueada et al. [53]. This SNP influences the ratio between mRNA for full length and soluble form of CTLA-4. The results from our previous study indicated that this SNP, together with Jo31, is associated with variations in the levels of membrane and cytoplasmic CTLA-4 in CD4+ T lymphocytes in multiple sclerosis patients [54], thus enhancing the activity of T-cells.

In the present study we have not found the association between CT60 and Jo31 SNPs, which are in strong linkage disequilibrium, with susceptibility to prostate cancer.

Table 3 Distribution of haplotypes for following SNPs: *CTLA-4*c.49A>G, *CTLA-4*g.319C>T, *CTLA-4*g.*6230G>A (CT60), *CTLA-4*g.*10223G>T (Jo31), *CD28*c.17+3T>C and *CD28*c.-1042G>A in prostate cancer patients compared with controls

haplotype*	Case (freq)	Control (freq)	*p*	OR	[95%CI]
	N = 301	*N* = 301			
A C A T T A	111.04 (0.18)	109.41 (0.18)	0.81	1.04	[0.77–1.40]
A C A T T G	88.38 (0.15)	87.02 (0.15)	0.83	1.04	[0.75–1.44]
A C G G T G	33.58 (0.06)	30.15 (0.05)	0.62	1.14	[0.69–1.89]
A T G G C G	46.67 (0.08)	44.81 (0.07)	0.79	1.06	[0.69–1.63]
A T G G T G	21.18 (0.04)	14.29 (0.02)	0.22	1.53	[0.77–3.02]
G C G G T A	52.47 (0.09)	58.53 (0.10)	0.60	0.90	[0.61–1.33]
G C G G T G	154.59 (0.26)	170.73 (0.28)	0.35	0.88	[0.68–1.15]

Global Chi^2 = 2.67, df = 6 (frequency < 0.03 in both control & case has been dropped)

Pearson's *p* value is 0.85

*The order of SNPs in estimated analysis of haplotypes frequency: *CTLA-4*c.49A>G, *CTLA-4*g.319C>T, *CTLA-4*g.*6230G>A (CT60), *CTLA-4*g.*10223G>T (Jo31), *CD28*c.17+3T>C and *CD28*c.-1042G>A

Interestingly, the association of the both SNPs with another urinary cancer – clear cell renal cell cancer was found by our group [55]. An increasing number of studies have been devoted to the association between the CT60 and Jo31 SNPs and cancers. The latest meta-analysis indicated that CT60 SNP is associated with the risk of breast cancer and cervical cancer especially in Asian population [56].

The functional roles of *CD28*c.17+3 T>C and *CD28*c.-1042G>A have not been clearly established. It is known that the *CD28*c.17+3T>C polymorphic site is situated within a region where a splice receptor site for CD28 is present, while *CD28*c.-1042G>A has been shown to be associated with reduced metastases-free survival in melanoma patients [46]. No association was found in the present study in the univariate analysis between *CD28* gene polymorphisms (c.17+3T>C and c.-1042G>A) and susceptibility to PCa.

A limitation of this study is the relatively small group of patients, but this group is genetically homogenous, which was reflected in virtually identical frequencies of H-Y polymorphisms in different regions of Poland [57]. Since this is the first report on the association of the *CD28/CTLA-4* gene region and prostate cancer risk, additional studies will be important to confirm and extend our results.

In conclusion: The functional polymorphisms in *CTLA-4* gene, which influence the surface protein level, might be considered as potential low risk factors for PCa development, but keeping in view the correction for multiple hypothesis testing, our results should be treated with caution and require further studies on a larger group of patients.

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Authors Contributions LK conceived and designed the study, performed statistical analysis, interpreted the results, wrote the manuscript; KT conceived and designed the study, participated in drafting the manuscript; AT, AP, P-A E carried out molecular genetics study, analyzed and interpreted the data, performed statistical analysis; KT, AK, DJ, WA recruited the patients for the study, collected the medical data; RZ, FI analyzed and interpreted the medical data and revised the manuscript critically for disease association content. All authors read and approved the final manuscript.

Compliance with Ethical Standards

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

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