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ORIGINAL ARTICLE

CTLA4 exon1 A49G polymorphism in Slovak patients with rheumatoid arthritis and Hashimoto thyroiditis—results and the review of the literature

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Abstract Autoimmune thyroid diseases frequently overlaps with rheumatoid arthritis (RA). Among genetic factors, the role of the HLA antigens and CTLA4 gene polymorphisms in the overlapping has been suggested. The aim of this study was to investigate the alleles and genotypes frequency of the CTLA4 exon1 A49G polymorphism in Slovak patients with RA, Hashimoto thyroiditis (HT), both (RA + HT) and in healthy controls. Fifty-seven unrelated adults with RA, 57 patients with HT, 34 patients with both (RA + HT), and 51 normal subjects were studied. All were ethnic Slovaks living in the same geographical area. The CTLA4 exon1 A49G polymorphism was genotyped by using small amplicon melting analysis after real-time PCR. The CTLA4 49GG genotype and G allele frequency in the group with RA was not significantly higher in comparison with controls (10.53% vs. 9.8%, p=0.62, OR 1.39, 95% CI 0.35–5.74 and 39.47% vs. 34.31%, p=0.43, OR 1.25, 95% CI 0.72–2.18). The frequency of GG genotype was slightly but not significantly higher in patients with HT as compared with control group (19.3% vs. 9.8%, p=0.17, OR 2.27, 95% CI 0.67–8.45). However, the frequency of GG genotype and G allele in patients with both RA and HT was significantly higher than that in controls (29.41% vs. 9.8%, p=0.02, OR 4.49, 95% CI 1.20–18.54 and 51.47% vs. 34.31%, p=0.03, OR 2.02, 95% CI 1.08–3.81). The frequency of GG genotype of CTLA4 A49G gene polymorphism in Slovak patients with RA is not significantly higher in comparison to control group. However, carriers of GG genotype with RA may be susceptible to develop HT.

Keywords A49G CTLA4 polymorphism · Autoimmune thyroid disease · CTLA4 gene · Hashimoto thyroiditis · Rheumatoid arthritis

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Introduction

It has been widely accepted that both organ-specific and non-organ-specific autoimmune diseases including rheumatoid arthritis (RA), Sjögren's syndrome, and systemic lupus erythematodes may often become associated with AITD, such as HT or Graves disease (GD). Thyroid autoimmunity may either precede or follow the establishment of systemic autoimmune diseases [1]. An increased frequency of thyroid autoimmunity was found in many larger cohorts among RA patients. Approximately 10–30% of RA patients have evidence of thyroid dysfunction and anti-thyroid autoantibodies as usually present in higher percentage of patients with RA than in the general population [2, 3]. AITD and RA are autoimmune diseases mediated by self-reactive T cells and other cells of the adaptive and innate immune systems. Sibling and twin studies indicate a major genetic component of the familial clustering of these common diseases with the major susceptibility loci in the human leukocyte antigen region (HLA) [4, 5]. Recent genome-wide association studies have successfully identified many single-nucleotide polymorphisms (SNPs) outside the HLA complex associated with common disease susceptibility [6]. The concept of other susceptibility genes than

HLA haplotypes common to different autoimmune diseases is now firmly established with previous studies demonstrating overlap of loci conferring susceptibility to RA and AITD in some ethnic populations. Examples include CTLA4, STAT4, PTPN2, CD226/Gly307Ser in both diseases, RA and AITD. These observations suggest that these diseases share common genetic susceptibility factors. This hypothesis is supported by findings of many genetic studies [7, 8].

Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is a member of the immunoglobulin superfamily and a structural homologue of CD28, but plays a negative regulatory role in T-cell response. CTLA4 has been reported to be an important negative regulator of various autoimmune diseases such as RA, GD, HT, type 1 diabetes mellitus (T1D), and multiple sclerosis [9]. The human CTLA4 gene is located on chromosome 2q33 and encodes the CTLA4 molecule which is involved in the control of T-cell proliferation and accumulation of IL-2, and mediates T-cell apoptosis by binding the B7 molecules on antigen-presenting cells constituting the B7/CD28-CTLA4 co-stimulatory pathway of T-cell activation. Thus, the CTLA4 gene is a strong candidate for susceptibility to T cell-mediated autoimmune diseases. The human CTLA4 gene is known to contain several polymorphisms; some of them are related to autoimmunity and autoimmune diseases.

The following three CTLA4 polymorphisms have been the most frequently studied to date. The first was a dinucleotide repeat polymorphism in exon 3 [10]. At least 23 different alleles ranging from 7 to 30 AT repeats have been identified. The second polymorphism was identified in the promoter sequence with a C to T transition at position-318 of the promoter sequence [11]. The third one is a G to A transition at position 49 of exon 1. The SNP led to an alanine to threonine amino acid substitution of codon 17 of the leader peptide (12). CTLA4 gene exon1 A49G polymorphism has been shown to be associated with various autoimmune disorders including AITD and RA [13–15]. However, the results were not consistent in different ethnic populations. The studies of CTLA4 gene polymorphism in RA are still limited and inconclusive [14, 16–24].

Therefore, aim of this study was to assess the occurrence of polymorphism CTLA4 exon1 A49G in patients with RA, HT and in the group with both RA and HT as well as control adults, all of whom were Slovak living in East-Slovakia region.

Materials and methods

We genotyped 199 subjects (148 patients and 51 healthy controls) living in the same geographical area—in Eastern Slovakia. The first group of these patients consisted of 57 unrelated adults who fulfilled the 1987 revised criteria for RA (46 females and 11 males, mean age 56.02±10.67 years, range 29–74 years).

The second group of patients consisted of 57 unrelated adults with Hashimoto thyroiditis (57 females, mean age 46.96±12.73 years, range 18–69 years). HT was postulated on the base of ultrasound picture and positivity of antibodies against thyreoglobulin (TgAb) and/or thyreoperoxidase (TPOAb). The third group consisted of 34 unrelated adults with the known coexistence of both RA and HT (34 females, mean age 57.97±3.61 years, range 29–74 years).

The healthy control group consisted of 51 unrelated ethnically matched healthy subjects who were randomly selected of community volunteers in the same district, free of any clinical evidence of autoimmune diseases. The median age at onset was 42.81±15.96 years (range 19–78 years). The study was approved by the local ethics committee and all subjects gave informed consent.

Genotyping

Genomic DNA was extracted from whole blood using a GenElute Blood GenomicDNA kit (Sigma-Aldrich), and was resuspended in water to bring concentration up to approximately 20 ng/μl. Recently, a rapid and inexpansive closed-tube assay using small amplicon melting analysis after real-time polymerase chain reaction (PCR) was described. This approach was adapted for genotyping A49G SNPs in the CTLA4 gene in our laboratory. Heterozygotes are identified by a change in melting curve shape, and different homozygotes are distinguished by a change in melting temperature (Tm). The PCR was performed in glass capillaries on a LightScanner32 instrument (Idaho Technology Inc. Salt Lake City, USA) in a 15-μl of reaction volume. Master mix contained: 1× BioThermAB™ buffer (GenCraft, Munster, Germany), 1x LCGreen $^{Plus+}$ (Idaho Technology Inc.), 250 μM dNTP (Jena Bioscience, Jena, Germany), 0.6 μM forward-primer: 5′-CGGCACAAGGCTCAGCTGAA-3′, 0.6 μM, reverse-primer: 5′-AGGAGAGTGCAGGGCCAGGTC−3′, 3 mM $MgCl_2$ (Idaho Technology Inc), 250 μg/ml BSA (Fermentas, Burlington, Canada) 1U BioThermAB™ polymerase (GenCraft) and approximately 20 ng DNA. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 45 cycles at 95°C for 10 s, 53°C for 15 s, and 72°C for 15 s. Amplification was immediately followed by melting analysis with a denaturation at 95°C for 30 s and renaturation at 40°C for 1 min. Data were acquired over the 75–95°C range at the thermal transition rate of 0.05°C/s. The specificity of the assay was comparable to that obtained by a PCR-RLFP method employing PCR with forward primer (5′-AGTCCTTGATTCTGTGTGGGT−3′) and reverse primer

(5′-TTGCAGAAGACAGGGATG-3′). The resulting DNA fragment (209 bp) was digested with BbvI (New England BioLabs).

Statistical analysis

The effects of CTLA4 genotypes and alleles on systemic autoimmune diseases were estimated by odds ratio (OR) with 95% CIs, and two-sided p calculated by the chi-square test. These computations were undertaken using the statistical software OpenEpi Ver. 2.3 (http://www.openepi.com). Evaluation of the Hardy–Weinberg equilibrium was performed by comparing observed and expected genotypes, using the chi-square test. Statistical significance was defined as $p<0.05$.

Results

CTLA4 exon1 A49G polymorphism was successfully genotyped in 148 patients and 51 healthy controls. Tables 1 and 2 show the genotype and allele distribution of CTLA4 A49G polymorphism among studied groups. The genotype frequencies among controls and patients were in Hardy–Weinberg equilibrium. The frequency of GG genotype was not significantly higher in RA patients as compared with controls (10.53% vs. 9.8%, $p=0.62$, OR 1.39, 95% CI 0.35–5.74). The frequency of GG genotype in patients with HT was slightly but not significantly higher as compared with the control group (19.3% vs. 9.8%, $p=0.17$, OR 2.27, 95% CI 0.67–8.45). However, patients with both RA and HT had significantly higher prevalence of the GG genotype than those in control group (29.41% vs. 9.8%, $p=0.02$, OR 4.49, 95% CI 1.20–18.54). The frequency of the G allele was significantly higher in patients with both disorders as compared with healthy controls (51.47% vs. 34.31%, $p=0.03$, OR 2.02, 95% CI 1.08–3.81).

When compared to patients with both RA and HT and the group of patients with RA only, the frequency of GG genotype was higher in the group of patients with the coexistence of both diseases (RA and HT); however, it reached only borderline statistical significance (29.41% vs. 10.53%, $p=0.06$, OR 3.24, 95% CI 0.89–12.56) Table 2.

Table 1 CTLA4 A49G exon 1 polymorphism in patients with RA, HT, RA + HT and controls

	RA	Control	OR	95%CI	p (two-tail)
	n=57 (%)	n=51 (%)			
Genotype					
AA	18 (31.58)	21 (41.18)	1.0		
AG	33 (57.89)	25 (49.02)	1.53	0.67–3.52	0.30
GG	6 (10.53)	5 (9.80)	1.39	0.35–5.74	0.62
Allele frequencies					
A	69 (60.53)	67 (65.69)	1.0		
G	45 (39.47)	35 (34.31)	1.25	0.72–2.18	0.43
	HT	Control	OR	95%CI	p (two-tail)
	n=57 (%)	n=51 (%)			
Genotype					
AA	20 (35.09)	21 (41.18)	1.0		
AG	26 (45.61)	25 (49.02)	1.09	0.48–2.51	0.83
GG	11 (19.30)	5 (9.80)	2.27	0.67–8.45	0.17
Allele frequencies					
A	66 (57.89)	67 (65.69)	1.0		
G	48 (42.11)	35 (34.31)	1.39	0.80–2.43	0.24
	RA + HT	Control	OR	95%CI	p (two-tail)
	n=34 (%)	n=51 (%)			
Genotype					
AA	9 (26.47)	21 (41.18)	1.0		
AG	15 (44.12)	25 (49.02)	1.39	0.50–3.96	0.51
GG	10 (29.41)	5 (9.80)	4.49	1.20–18.54	0.02
Allele frequencies					
A	33 (48.53)	67 (65.69)	1.0		
G	35 (51.47)	35 (34.31)	2.02	1.08–3.81	0.03

Table 2 CTLA4 A49G exon 1 polymorphism in patients with RA, HT, and RA + HT

	RA	HT	OR	95%CI	*p* (two-tail)
	n=57 (%)	*n*=57 (%)			
Genotype					
AA	18 (31.58)	20 (35.09)	1.00		
AG	33 (57.89)	26 (45.61)	1.41	0.62–3.23	0.41
GG	6 (10.53)	11 (19.30)	0.61	0.18–2.00	0.40
Allele frequencies					
A	69 (60.53)	66 (57.89)	1.00		
G	45 (39.47)	48 (42.11)	0.89	0.53–1.53	0.69
	RA + HT	HT	OR	95%CI	*p* (two-tail)
	n=34 (%)	*n*=57 (%)			
Genotype					
AA	9 (26.47)	20 (35.09)	1.00		
AG	15 (44.12)	26 (45.61)	1.28	0.46–3.64	0.63
GG	10 (29.41)	11 (19.30)	1.99	0.61–6.62	0.23
Allele frequencies					
A	33 (48.53)	66 (57.89)	1.00		
G	35 (51.47)	48 (42.11)	1.45	0.79–2.68	0.22
	RA + HT	RA	OR	95%CI	*p* (two-tail)
	n=34 (%)	*n*=57 (%)			
Genotype					
AA	9 (26.47)	18 (31.58)	1.00		
AG	15 (44.12)	33 (57.89)	0.91	0.33–2.58	0.85
GG	10 (29.41)	6 (10.53)	3.24	0.89–12.56	0.06
Allele frequencies					
A	33 (48.53)	69 (60.53)	1.00		
G	35 (51.47)	45 (39.47)	1.62	0.88–2.99	0.11

Discussion

Our present study showed that A49G polymorphism of CTLA4 gene could influence the development of HT in Slovak patients with RA. There was evidence that the GG genotype frequency was significantly higher in the group with both HT and RA to that in the control group (29.41% vs. 9.8%, p=0.02, OR 4.49, 95% CI 1.20–18.54) and to the group with RA (29.41% vs. 10.53%, p=0.06, OR 3.24, 95% CI 0.89–12.56) reaching borderline statistical significance. The G allele was significantly more common in patients with both diseases than in healthy controls (51.47% vs. 34.31%, p=0.03, OR 2.02, 95% CI 1.08–3.81). These results suggest that the prevalence of GG genotype and G allele of CTLA4 A49G gene polymorphism is higher in patients with the coexistence of HT with other autoimmune disorder, namely in RA patients as was demonstrated in our study. In agreement with previous studies in European cohorts we did not demonstrate a higher frequency of the GG genotype and G allele in RA as compared with controls.

In the current literature, studies on the polymorphism of CTLA4 exon1 A49G in RA have shown conflicting results mostly depending on ethnicity. Four studies [14, 16–18] found an association between this polymorphism and RA independent of HLA-DRB1 alleles whereas seven studies did not find such association [15, 19–24]. Three studies further demonstrated that the association was more significant with respect of some specific HLA DRB1 alleles [16, 17, 20]. In order to look for ethnic effect Shizhong Han et al., 2005 performed subgroup meta-analysis in populations of European and Asian descent [25]. Meta-regression analysis was also performed to explore the possible heterogeneity between the two subgroups. Ten studies (11 comparisons) with the CTLA4 exon1 A49G genotyping on 2,315 patients with RA and 2,536 controls were selected for their meta-analysis. Overall, the fixed-effects odds ratio for the G versus A allele was 1.11 (p=0.02, 95% CI 1.02–1.21) with no between-study heterogeneity. Subgroup and meta-regression analysis according to the ethnicity (European or Asian) demonstrated different scenarios concerning the CTLA4 exon1 A49G polymorphism's role in RA susceptibility for the two different subgroups. No effect of G on susceptibility was seen in European descent. However, there was a significant association in Asian descent under both fixed and random-effect models. The results of the

meta-analytic study suggest that CTLA4 exon1 49G allele would not be a risk factor for RA in Europeans but might play role in RA susceptibility for Asians [25]. This finding is in accordance with our results demonstrating no significant difference in the distribution of A49G genotypes and alleles between RA patients and control subjects (Tables 1 and 2).

Moreover, in our study, the G/G genotype of CTLA4 A49G polymorphism in the group of patients with HT tended to be more frequent than in controls although it did not reach statistical significance, possibly as a result of the small sample size of HT patients. In general, the genotypes and alleles frequencies were not significantly different between groups of HT, RA, as well as control subjects.

Despite a strong epidemiological evidence for genetic predisposition, only HLA region and gene for CTLA4 have up to now been identified to confer the susceptibility to HT [26]. Many studies confirmed the association between the promoter, exon 1 and 3′untranslated region CTLA4 gene polymorphisms and AITD, including Graves' disease and HT [27, 28]. In addition, there are studies verifying relation between CTLA4 gene and thyroid autoantibody (TAb) production [29, 30]. In the study of Zaletel K et al., authors provide evidence that CTLA4 A49G exon 1 polymorphism is associated with TAb status in patients with HT [29]. In the study performed by Shizong et al., the association of CTLA exon 1 A49G polymorphism with GD risk in 261 unrelated Chinese GD patients and 196 age and sex-matched healthy controls were investigated. GD patients presented with a significantly higher portion of GG genotypes and G allele compared with control subjects. There was evidence that the presence of 49G allele resulted in increased susceptibility to GD (OR=1.48, 95% CI 1.12–1.94) [31]. At present, there is no obvious explanation why patients with specific CTLA4 polymorphisms like A49G more frequently fail to maintain immune tolerance to thyroid antigens. The autoimmune response may be influenced by an altered CTLA antigen expression and function in subjects carrying specific CTLA4 genotypes. The effect of G allele on T cell function was firstly shown by Kouki et al., 2000, who found GG genotype to be associated with reduced inhibitory function of CTLA4 on T-cell proliferation after stimulation with an allogenic cell line both in patients with Graves disease and in healthy controls [32] which was confirmed by others [33, 34]. However, when Xu et al., 2002 examined the recombinant human CTLA4, there was no difference between the function of CTLA4 transcripts encoded by A or by G allele [35]. Thus, as this report seems to indicate, A49G polymorphism may be in the linkage disequilibrium with another, so far undiscovered polymorphism causes alteration in CTLA4 expression and function.

There are only two reports comparing the frequency of the GG genotype of A49G polymorphism of CTLA4 gene in patients with both RA and HT to the frequency of this genotype in controls. Our finding of the higher frequency of GG genotype and G allele in patients with the coexistence of both RA and HT is in accordance with the study by Vaidya et al. reporting [15] that there was an association between the CTLA4 G allele and RA, but noted that this was largely explained by individuals with coexisting autoimmune endocrinopathies. In this study, the frequency of the G allele at CTLA4 A/G was significantly increased in probands with early RA compared with controls [43% vs. 36%; p=0.028, OR 1.35, 95% confidence interval (CI) 1.01–1.82]. Most of this increased frequency was attributable to RA individuals with coexisting autoimmune thyroid disease or type 1 diabetes (58% vs. 36% in controls; p=0.005, OR 2.50, CI 1.29–4.84). The frequency of the G allele in RA patients without autoimmune endocrinopathy was 40%, which was not significantly different from that in controls (p=0.140) and this is in agreement with our results. We suggest that the higher frequency of G allele and GG genotype in patients with coexistence of both diseases might be due to HT being well documented in studies mentioned above [15, 28, 29].

In conclusion, our result suggests that also patients with GG genotype of CTLA4 exon1 A49G gene polymorphism with RA may be susceptible to develop HT. Moreover, we did not confirm the higher frequency of the GG genotype as well as G allele in Slovak patients with RA than that in control group.

Disclosures None.

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