



***CD40* rs4810485 *T>G* polymorphism and susceptibility to ankylosing spondylitis in the Latvian population**

J. Zepa^{1,2,3}, L. Silamiķele⁴, I. Bulina^{1,2,3}, V. Lavrentjevs¹, I. Trapina⁵, J. Klovinš⁴, D. Andersone^{1,3}, A. Lejnieks^{2,6} and L. Nikitina-Zake⁴

¹ Center of Rheumatology, Pauls Stradins Clinical University Hospital, Riga, Latvia

² Riga Stradins University, Riga, Latvia

³ Faculty of Medicine, University of Latvia, Riga, Latvia

⁴ Biomedical Research and Study Centre of Latvia, Riga, Latvia;

⁵ Genomics and Bioinformatics laboratory in the Institute of Biology, University of Latvia, Salaspils, Latvia

⁶ Riga East Clinical University Hospital, Riga, Latvia

Corresponding author: J. Zepa

E-mail: julija.zepa@inbox.lv

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ABSTRACT. Ankylosing spondylitis (AS) is a potentially disabling form of a systemic chronic inflammatory arthritis affecting mainly the axial skeleton, with or without extraspinal manifestations. The genetic basis of AS is partly known. Moreover, many autoimmunity-related genes have pleiotropic effects. Multiple functional polymorphisms in the genes encoding the tumor necrosis factor (TNF) superfamily of cytokines, their receptors, and signaling proteins, are associated with susceptibility to autoimmune diseases. These arguments prompted us to conduct a study evaluating a possible association of single nucleotide polymorphism (SNP) rs4810485 of the *CD40* gene, found previously to be involved in other inflammatory diseases, with susceptibility to AS in the Latvian population; 98 AS patients from the Center of Rheumatology of Pauls Stradins Clinical University Hospital were selected as the case group and 154 ethnically related healthy subjects from the Genome Database of the Latvian Population were included as a control group.

CD40 gene rs4810485 polymorphism was tested by a TaqMan Pre-Designed SNP Genotyping Assay. In the case of *CD40* gene rs4810485 polymorphism, the minor allele is *T*. Compared to the control subjects, the case group had a higher frequency of the minor allele *T* (28.6% vs. 17.5%; $p=0.00345$). The *T* allele was the risk allele for disease onset (OR 1.88 (95% CI 1.23-2.88)). The relationship between the disease and genotypes was of moderate significance ($V=0.20$). As for genotypes, *GT* and *TT* were the susceptibility genotypes for AS (respectively OR 2.42 (95% CI 1.38 – 4.25) and 1.94 (95% CI 0.71-5.32)). The *GG* genotype had a protective feature (OR 0.43 (95% CI 0.25 – 0.73)). A significant difference was not found in the analysis of the SNP alleles and genotype distribution in the peripheral arthritis ($p=0.85$ and $p=0.86$, respectively) and uveitis ($p=0.47$ and $p=0.3$, respectively) subgroups of AS patients. The study data showed that *CD40* gene rs4810485 polymorphism is associated with risk of AS.

Key words: Ankylosing spondylitis; *CD40* gene; Gene polymorphism; Disease susceptibility

INTRODUCTION

Ankylosing spondylitis (AS) is a potentially disabling form of a systemic chronic inflammatory arthritis that mainly affects the axial skeleton through inflammation and structural damage, with or without extraspinal manifestations, such as peripheral arthritis, enthesitis, dactylitis, uveitis etc. (de Winter et al., 2016). Clinically the most prominent and disabling symptom is inflammatory back pain manifested during the night and early morning, with a following period of prolonged stiffness. The consequence of inflammation is structural damage. Aggressive and long-standing AS results in ankylosis of sacroileal joints and bamboo-like spine (bony bridges made from the fusion of syndesmophytes and ossification of ligaments) manifested as a significant reduction of spinal mobility and thus causing functional disability. This disease usually affects young male and female patients during their most productive years (Rudwaleit et al., 2009). Overall, estimates of the prevalence of AS are between 0.2% and 1.2%, with most of these data coming from Europe (Sieper et al., 2006). Early diagnosis of chronic systemic rheumatic diseases is important in order to improve the long-term outcome of systemic inflammation. Treatment of AS has progressed from symptom relief to pathogenetic treatment using disease modifying antirheumatic drugs, especially tumor necrosis factor (TNF) alpha inhibitors. However, not all patients develop remission, demonstrating a need for more studies. The next level in the care of AS patients is cure of the disease and, more importantly, prevention of its development. One of the means to achieve this is to understand the genetic basis of this disease.

Based on family and twin studies, AS is known to be highly heritable, with >90% of the risk of developing the disease determined genetically (Brown, 2008). There is considerable data suggesting that knowledge about *HLA-B*27* (human leukocyte antigen B27), a gene encoded in major histocompatibility complex (MHC), though important, is not

sufficient to explain the genetic epidemiology of AS (Brown et al., 2000). *HLA-B*27* is found in 90-95% of AS patients (Khan, 2000). In a European population study, AS was found in 1.3% of *HLA-B*27* positive individuals in the population at large and in 21% of *HLA-B*27* positive relatives of *HLA-B*27* positive AS patients, demonstrating a 16-fold risk of AS in *HLA-B*27* positive relatives compared with *HLA-B*27* positive individuals in the general population (van der Linden et al., 1984). Thus, only a small proportion of *HLA-B*27* positive individuals in the general population develop AS, suggesting that there are other susceptibility factors (Brown et al., 2000) and arguing against AS being a monogenic disease.

Based on data published in 2013 by the International Genetics of Ankylosing Spondylitis Consortium (IGAS), in addition to *HLA-B*27* alleles and 12 loci (*ANTXR2*, *CARD9*, *ERAP1*, *IL12B*, *IL23R*, *KIF21B*, *PTGER4*, *RUNX3*, *TBKBP1*, *TNFRSF1A* and chromosomes 2p15 and 21q22) associated with AS that have been identified previously in populations of European ancestry, 13 new risk loci (nearby genes *IL6R*, *FCGR2A*, *UBE2E3*, *GPR35*, *BACH2*, *ZMIZ1*, *NKX2-3*, *SH2B3*, *GPR65*, *IL27-SULT1A1*, *NOS2*, *TYK2*, *ICOSLG*) and 12 additional AS-associated haplotypes at 11 loci were discovered. These findings highlight the role of some major biological pathways in the pathogenesis of AS, including the IL-23 pathway, gut immunity, T-lymphocyte differentiation or activation, and peptide processing before HLA class I presentation. In total, 24.4% of the heritability of AS is now explained: 4.3% from loci other than *HLA-B* and 20.1% due to *HLA-B*27* itself (IGAS, 2013).

The genetic basis of AS is partly known. Moreover, many autoimmunity related genes have pleiotropic effects. Multiple functional polymorphisms in the genes encoding tumor necrosis factor (TNF) superfamily of cytokines, their receptors and their signaling proteins are associated with the susceptibility to autoimmune diseases (Croft and Siegel, 2017). These arguments prompted us to conduct a study evaluating the role of the *CD40* gene, which has been tested previously in relation to other inflammatory diseases, including rheumatic diseases (Lee et al., 2015), in susceptibility to AS in the Latvian population.

This gene encodes CD40 protein - a member of the tumor necrosis family of transmembrane glycoproteins identified in B cells, monocytes, dendritic cells, endothelial and epithelial cells. The CD40 protein is a receptor on antigen-presenting cells of the immune system and is essential for mediating a broad variety of immune and inflammatory responses (Benveniste et al., 2004). Recently, a number of polymorphisms in the gene encoding CD40 have been identified and a relationship between these *CD40* gene polymorphisms and risk of different autoimmune and inflammatory diseases, such as Graves' disease, systemic lupus erythematosus, rheumatoid arthritis, atherosclerosis has been reported (Chen et al., 2015). A possible association between *CD40* gene polymorphisms and AS has not been studied previously.

The objective of this study was to investigate the association of SNP rs4810485 of the *CD40* gene in AS patients compared to controls in the Latvian population.

MATERIAL AND METHODS

Patients

Ninety-eight Caucasian patients fulfilling the modified New York criteria for AS (van der Linden et al., 1984) were included in a cross-sectional study. The data presented

here focus on patients with adult onset predominantly axial disease who were included in the study between October 2011 and February 2016 in the Center of Rheumatology of Pauls Stradins Clinical University Hospital (outpatient and inpatient department). The inclusion criteria were age greater than 18 years during the onset of the symptoms and fulfilling the modified New York criteria for AS. The exclusion criteria were other chronic or autoimmune inflammatory arthritis syndromes (for example, rheumatoid arthritis), systemic lupus erythematosus, psoriasis, multiple sclerosis, inflammatory bowel disease, type 1 diabetes, Graves' disease, moderate or severe cardiac, pulmonary, renal and/or hepatic insufficiency, acute or chronic infection, depression or other psychiatric diseases diagnosed and/or treated by psychiatrists, CNS disorders recorded in medical reports, drug and/or alcohol abuse.

The following variables were chosen for overall characteristics of AS patients: age, duration of the disease, the age at the onset of AS, disease activity evaluation using specific indices such as BASDAI (the Bath Ankylosing Spondylitis Disease Activity Index) (Garrett et al., 1994) and ASDAS (Ankylosing Spondylitis Disease Activity Score) (Machado et al., 2011), functional (BASFI – the Bath Ankylosing Spondylitis Functional Index; 0 - 10) (Calin et al., 1995) and metrological (BASMI 3-point answer scale – the Bath Ankylosing Spondylitis Metrological Index) (Jenkinson et al., 1994) indices. BASDAI consists of six patient-reported outcomes, and has an overall score from 0 to 10; evaluation <4 represents inactive disease. ASDAS was calculated using a specific formula, including evaluation of back pain (the second item of BASDAI), duration of morning stiffness (the sixth item of BASDAI), patient global disease activity evaluation, peripheral pain/swelling (the third item of BASDAI) and C-reactive protein (CRP). Elevated CRP was considered if CRP ≥ 5 mg/L. The presence or history of peripheral arthritis and uveitis was taken into account for the analysis of AS subgroups.

Control group

DNA from 154 ethnically related healthy subjects from the Genome Database of the Latvian population were included as a control group. The controls were matched for age and area of residence.

Genotyping of CD40 rs4810485 polymorphism

An aliquot of 9 ml of venous blood was collected from each subject in tubes containing ethylenediamine tetraacetic acid. DNA was isolated by standard phenol/chloroform extraction method. Extracted DNA was diluted to 10 ng/ μ l and used for genotyping.

Samples were genotyped by Real Time PCR using TaqMan Pre-Designed SNP Genotyping Assay for rs4810485 (Thermo Fisher Scientific).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested in the control group using a heterozygosity index and the Chi-square test. Allele and genotype frequencies of the SNP were detected by direct calculation. The Chi-square test was used to compare the

differences in allele and genotype frequencies of the SNP between case and control groups. The calculation was performed with SPSS 23.0 software. P values <0.05 were considered statistically significant. The association between the allele and genotype with the disease was detected using Cramer's V: very weak: <0.10; weak: 0.10 – 0.19; moderate: 0.20 – 0.29; strong: 0.30 – 0.40; very strong: 0.40 – 0.50; almost perfect: >0.50. Odds ratios (OR) and 95% confidence intervals (95% CI) were used to represent the relative risk of AS. An additive model was used for rare allele/genotype and a multiplicative model for frequent genotype. OR value >2 or <0.5 means the chance of getting the disease is very high, but OR >3 or <0.33 – clinically meaningful.

Ethical considerations

Ethical approval was obtained for this study from the local ethics committee at Pauls Stradins Clinical University Hospital and the Central Medical Ethics Committee of Latvia. Written informed consent was obtained from all the patients in accordance with the principles of the Declaration of Helsinki.

RESULTS

We studied 98 AS patients and 154 healthy control subjects. Table 1 shows the overall characteristics of AS patients (males 98%) covering the mean age at disease onset (26.33 years), disease duration (13.79 years), disease activity evaluation (BASDAI 4.72; ASDAScrp 3.01), functional impairment level (BASFI 3.72) and spinal mobility evaluation (BASMI 3.89). Among all patients 58% had peripheral arthritis and 34% had uveitis, reported at any time during the course of the disease.

Table 1. Characteristics of ankylosing spondylitis (AS) patients.

Variable	Mean (SD)
Age (years)	40.17 (9.44)
Duration of the disease (years)	13.79 (8.29)
Age at the onset of AS (years)	26.33 (6.82)
BASDAI (0-10)	4.72 (2.26)
ASDAScrp	3.01 (1.38)
BASFI (0-10)	3.72 (2.69)
BASMI (0-10)	3.89 (2.60)
Peripheral arthritis (%)	58
Uveitis (%)	34

(Note: BASDAI-the Bath Ankylosing Spondylitis Disease Activity Index, ASDAScrp-Ankylosing Spondylitis Disease Activity Score, using CRP-c-reactive protein, BASFI-the Bath Ankylosing Spondylitis Functional Index, BASMI-the Bath Ankylosing Spondylitis Metrological Index)

Genotype and allele distribution of CD40 gene rs4810485 polymorphism is shown in Table 2. In the case of CD40 gene rs4810485 polymorphism, the minor allele was T. In addition, compared to the control subjects, the cases had a higher frequency of minor allele T (28.57% vs 17.53%; $p=3.45 \times 10^{-3}$; Table 2), though the relationship between the disease and the allele distribution was weak ($V=0.13$). The T allele showed a higher frequency when patients were stratified for age at the onset of the disease (OR_a 1.88 (95% CI 1.23-2.88)). All three genotypes were found at the rs4810485 locus in our study group; the most frequent was the GG genotype. Differences in genotype frequencies were significant (Table 2): frequency of GG genotype in AS group was 51% vs 71% in controls, GT genotype 41%

vs 23%, TT genotype 8% vs 6%, respectively ($p=6.7 \times 10^{-3}$). The most frequent genotype in the control group was GG, but in the case group the distribution between GG and GT genotypes was more similar than in the control group. The relationship between the disease and genotypes was of moderate significance ($V=0.20$). GT and TT were the susceptibility genotypes for AS (respectively OR_a 2.42 (95% CI 1.38 – 4.25); 1.94 (95% CI 0.71-5.32)). Moreover, the homozygote genotype GG had a protective effect (OR_m 0.43 (95% CI 0.25 – 0.73)). A significant difference was not found in the analysis of SNP alleles and genotype distribution between the peripheral arthritis ($\chi^2 = 0.03$, $p=0.85$ and $\chi^2=0.30$, $p=0.86$, respectively) and uveitis subgroups ($\chi^2 = 0.51$, $p=0.47$ and $\chi^2=2.38$, $p=0.30$, respectively) of AS patients.

Table 2. Comparison of allele and genotype frequencies of CD40 rs4810485 in the ankylosing spondylitis case and control groups.

Allele/genotype	Cases (n=94) (%)	Controls (n=154) (%)	χ^2	p	OR (95% CI)
G	71.43	82.47	8.55	3.45×10^{-3}	-
T	28.57	17.53			1.88 (1.23-2.88)
GG	51.02	70.78	10.22	6.70×10^{-3}	-
GT	40.82	23.38			2.42 (1.38-4.25)
TT	8.16	5.84			1.94 (0.71-5.32)

(OR – odds ratio, CI – confidence interval)

DISCUSSION

The study data characterizing the AS population underline the clinical significance of the impact of the disease on the quality of life: the results show high disease activity with functional impairment and spinal mobility restrictions; moreover, many patients had extraspinal manifestations such as peripheral arthritis and uveitis. All the symptoms are seen in relatively young subjects of reproductive and working age. Therefore it is extremely important to improve knowledge about the pathogenetic, clinical, radiological and genetic aspects of the disease to avoid longstanding undiagnosed cases with irreversible structural bone damage.

AS is a multigenetic disease. Several genes have been indicated as candidate genes contributing to AS onset. The CD40 gene has been widely investigated in different autoimmune diseases, including some rheumatic diseases. At present, CD40 rs4810485 G/T polymorphism is one of the most widely investigated polymorphisms that is closely related to susceptibility to systemic lupus erythematosus and rheumatoid arthritis (Lee et al., 2015). So far, to our knowledge, no research on the CD40 gene rs4810485 G/T polymorphism for association with AS has been performed. Our study showed that there is an association of CD40 gene rs4810485 G/T polymorphism with AS risk. GT and TT genotypes and T allele of rs4810485 are associated with increased risk of AS (OR_a 2.42 (95% CI 1.38 - 4.25), OR_a 1.94 (95% CI 0.71-5.32) and OR_a 1.88 (95% CI 1.23 - 2.88), respectively). We found that the homozygous GG genotype of rs4810485 may be a protective factor related to the development of AS (OR_m 0.43 (95% CI 0.25 - 0.73)).

As is widely recognized, gene polymorphisms frequencies vary in different populations and geographic regions, and the genetic distribution of populations in different regions is not identical, thus leading to frequency differences (Wang et al., 2015). Possible variations between ethnic groups should be taken into account and results should be examined accordingly. According to information in the NCBI's SNP database (dbSNP),

which analyzes *CD40* rs4810485 in different populations, the major allele is *G* and the most frequent genotype is *GG*.

The protein encoded by this gene is involved in the inflammatory process of several autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus. To a lesser extent it has been studied in AS. CD40 - a TNF receptor superfamily member expressed by immune and nonimmune cells has multipotent immunomodulatory functions. CD40 interacts with specific ligands that mediate T-dependent B cell responses and provide efficient T cell priming. Thus, CD40 is a likely candidate to play a role in autoimmune diseases in which activated T and B cells cause pathology (Peters et al., 2009). Diseases in which CD40 has a significant pathogenic role include autoimmune thyroiditis, type 1 diabetes, multiple sclerosis, psoriasis, inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus (Peters et al., 2009). Few publications have examined AS and CD40. One of them describes an increased risk of atherosclerosis in AS patients through analysis of many factors, including increased level of soluble CD40 ligand (Stanek et al., 2017).

There are also data that CD154, the CD40 ligand, is overexpressed in T-lymphocytes in peripheral blood of AS patients and can be down-regulated by etanercept (a TNF alpha inhibitor) treatment, which suggests that CD154 might be involved in the inflammatory process of AS and is a potential biomarker to monitor AS disease activity and treatment efficacy (Lin Q et al., 2010). It is known that TNF inhibitors are among the most effective protein-based drugs for reducing inflammation in AS. However, some AS patients are anti TNF treatment non-responders (Navarro-Compán et al., 2017). An open question is whether some patients do not respond to the TNF alpha inhibitors because several other TNF superfamily molecules are also active. In addition to TNF, the TNF superfamily, including CD40, comprises other ligand-receptor combinations that might participate in the pathogenesis of rheumatic diseases, including AS. So the TNF superfamily can be targeted to either regulate the immune response or to restore tolerance in rheumatic diseases (Croft and Siegel, 2017).

The course of this disease can vary considerably, including the presence of extraspinal manifestations. Previous studies dated from 2004 till 2016 have reported 18 – 58% prevalence of peripheral arthritis (reported at any time during the disease course). The reported prevalence of uveitis occurring at some point in time during the course of AS varies from 22 – 37% (de Winter et al., 2016). Our data on the frequency of peripheral arthritis and uveitis is consistent with the published data of meta-analysis on extraspinal manifestations in AS (de Winter et al., 2016). Analysis of the SNP allele and genotype distribution did not show significant differences in these subgroups of AS patients, thus highlighting the question about the genetic basis of the disease itself and the genetic basis of variability of the disease's clinical manifestations.

We conclude that the *CD40* gene may play an important role in susceptibility to AS. Our results suggest that further studies with larger cohorts of AS patients would be useful and should be performed to explore the association of the *CD40* gene polymorphism with AS susceptibility.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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