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INTERLEUKIN 18 GENE PROMOTER POLYMORPHISMS IN LATVIAN PATIENTS WITH RHEUMATOID ARTHRITIS

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Interleukin 18 (IL-18) is a proinflammatory cytokine involved in the pathogenesis of rheumatoid arthritis (RA). There are controversial reports suggesting that IL-18 *promoter polymorphisms may be an independent marker of RA susceptibility. The aim of the present study was to determine whether polymorphisms of the* IL-18 *gene promoter in positions -607 (rs 1946519) and -656 (rs 1946518) are associated with RA, and its characteristics in the Latvian population. We examined 105 patients with RA diagnosed according to the criteria of the American College of Rheumatology. DNA and phenotypic data from a healthy control population was obtained from Genome Database of Latvian Population. Genotypes were obtained by direct sequencing. Single-nucleotide polymorphisms (SNPs) were studied and frequencies of alleles and genotypes were compared between patients and controls. A* P *value less than 0.05 was accepted as statistically significant. There were no significant differences in the distribution of alleles and genotypes between RA patients and the control group. The frequencies of IL-18-607C/A and -656G/T genotypes differed between patients and the control group in women (*P *= 0.084 and 0.097). Heterozygous genotypes -607CA and -656GT occurred more frequently in the RA group than in the control (*P *= 0.046,* P *= 0.060), and this difference was also significant for the only women groups (*P *= 0.041,* P *= 0.054). The heterozygous states -607CA and -656GT of* IL-18 *gene affect susceptibility to RA. On the basis of investigated* IL-18 *polymorphisms, female patients with RA seem to represent a separate disease subgroup.*

Key words: *RA, IL-18, SNP, susceptibility, genetics.*

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease, characterised by chronic synovitis and progressive joint destruction. The etiology of RA is multifactorial and includes a significant genetic component. Cytokines act as mediators of immune and inflammatory responses and play an important role in the pathophysiology of joint inflammation and destruction (Feldmann *et al*., 1996). A central feature of RA is a relative imbalance of cytokine production with a relative excess of proinflammatory molecules, compared with antiinflammatory mediators (Feldmann *et al*., 1996). It has been observed that the production of proinflammatory cytokines is markedly increased in serum and synovial fluid in RA patients. The expression levels of cytokines have a genetic background. Single nucleotide polymorphisms (SNPs), the most common genetic variations that have been identified in the cytokine genes promoter, may be responsible for variations in cytokine production. The successful therapeutic use of inhibitors of proinflammatory cytokines (TNF-α, IL-6) underlines the importance of these molecules in driving rheumatoid inflammation and tissue damage. However, non-responder or partial responder patients are not uncommon, and inflammatory disease can flare on discontinuation of treatment. Therefore, new therapeutic targets in RA should be investigated.

Interleukin 18 (IL-18), a member of the IL-1 superfamily, was first identified as an interferon-γ (IFN-γ) inducing factor (Okamura *et al*., 1995). It is widely expressed in human tissues (Dinarello *et al*., 1998). IL-18 in RA is produced predominantly by tissue macrophages (Gracie *et al*., 1999; Tanaka *et al*., 2001; Yamamura *et al*., 2001). In addition, mature IL-18 may also be released from dendritic cells (Gardella *et al*., 1999), neutrophils (Westphal *et al*., 2006) and endothelial cells (Yamaoka-Tojo *et al*., 2003).

IL-18 has a highly diverse range of biological function. IL-18 induces the synthesis of TNF-α, granulocyte-macrophage colony stimulating factor, nitric oxide, and chemokines by T cells and natural killer cells (Dinarello, 1999). IL-18 directly activates synovial macrophages, promotes

production of proinflammatory cytokines such as TNF-α and IL-1β (Gracie *et al*., 1999), induces the production of hemokines and expression of adhesion molecules by synovial fibroblasts (Morel *et al*., 2001; 2002), and stimulates angiogenesis (Park *et al*., 2001). IL18 indirectly stimulates osteoklastogenesis through upregulation of membrane bound receptor activator of NFkB ligand (RANKL) production from RA synovial T cells or through induction of TNF-α, IL-1β and IL-6 (Dai *et al*., 2004).

IL-18 has been found in synovial tissue, and enhanced levels of IL-18 were observed in the serum and in the joint of RA patients (Gracie *et al*., 1999). Serum and synovial fluid IL-18 levels as well as synovial tissue IL-18 expression were correlated with disease activity parameters (erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP), and Disease Activity Score (DAS 28)) (Yamamura *et al*., 2001; Joosten *et al*., 2003; Rooney *et al*., 2004; Petrovic-Rackov *et al*., 2006).

The *IL-18* gene maps to chromosome 11q22.2-q22.3 (Nolan *et al*., 1998). Several single-nucleotide polymorphisms (SNPs) in the promoter region in the *IL-18* gene are present, which may affect IL-18 expression. Allelic variants of cytokine genes associated with promoter gene region polymorphisms do not influence the protein amino acid sequence but can result in changes of cytokine production. The -607 C/A SNP is located at the binding sites for CREB transcriptional factors (cAMP response-element binding proteins), and therefore, mutations at this site can influence IL-18 expression and change the production of the cytokine (Giedraitis *et al*., 2001).

The aim of the present study was to examine two *IL-18* gene SNPs in positions -607 (rs 1946519) and -656 (rs 1946518) in the promoter region in relation to the RA susceptibility, activity and severity.

MATERIALS AND METHODS

Patient population. A total of 105 patients with established RA (women and men) according to the well-known revised criteria of the American College of Rheumatology (ACR) for RA (Arnett *et al*., 1988) were consecutively recruited into the study during a one-year period. Patients with RA were recruited from the outpatient and inpatient populations in the Rheumatology Department Clinic "Linezers", Rīga Eastern Hospital, Latvia. Informed consent was obtained from all patients.

Evaluation of the patients included a physical examination with a particular focus on the pattern of joint involvement and laboratory analyses. The following data were obtained from the RA patients: age of disease onset, number of tender (TEN) and swollen (SW) joints, visual analogue scale (VAS) of pain and disease activity, physician assessment of disease activity, presence and value of rheumatoid factor (RF) and anti-cyclic-citrullinated peptides (antiCCP) antibodies, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rentgenological (RTG) stage. The modified disease activity score DAS28 was calculated as described (Prevoo *et al*., 1995). Simple x-rays of the hands and feet were analysed using the Steinbrocker method (Pincus *et al*., 1997). Within the RA group, eight patients lacked evidence of joint erosions on the radiograph (stage I: nonerosive RA) and 97 patients had erosions (stages II–IV: erosive RA; 19 – stage II, 75 – stage III, and 3 – stage IV). Demographic, clinical, and laboratory characteristics of RA patients are summarised in Table 1.

A total of 242 healthy subjects with similar age and sex distribution were available for the study from Genome Database of Latvian Population (median 53, range 17–84 years, female 195 (81.3%), male 45 (18.8%)).

Genetic analysis. Genotypes were obtained by direct sequencing. Analysis of SNPs at the -607 and -656 positions of the *IL-18* gene were studied.

Genomic DNA was extracted from peripheral blood samples using the standard phenol-chloroform extraction method. The *IL18* promoter region harbouring SNPs at positions -607 and -656 was PCR amplified using forward 5'-ATTCAGGACTTCCCCTTCCT-3' and reverse 5'-CACTCTGCTCTTCAAACGTTACAT -3' primers. Amplicons were then purified using Sap-ExoI protocol and the sequences were obtained by direct sequencing of amplified products using forward PCR primer on a 3100 ABI prism DNA sequencer (Applied Biosystems).

Table 1

DEMOGRAPHIC, CLINICAL AND LABORATORY CHARACTERISTICS OF PATIENTS WITH RHEUMATOID ARTHRITIS

Characteristics	RA patients, n =105
Age (years)	58 (26–83)
Female sex (%)	89 (84.8)
Male sex (%)	16 (15.2)
RA onset	
before 50 y.o.	47 (44.8)
after 50 y.o.	58 (55.2)
Disease duration (years)	4.7 (0.1–49.4)
Swollen joint count (28)	6 (0–20)
Tender joint count (28)	8 (0–26)
VAS pain (mm)	49 (1–97)
VAS disease activity (mm)	50 (2–93)
VAS physician's assessment (mm)	34 (4–89)
ESR (mm/h)	26 (2–76)
CRP (mg/l)	6.90 (0.00–113.4)
DAS28 (ESR)	5.04 (0.93–7.77)
DAS28 (CRP)	4.5 (1.84–7.6)
Rheumatoid factor positivity	79 (76.7)
Rheumatoid factor negativity	24 (23.3)
AntiCCP positivity	58 (79.6)
AntiCCP negativity	15 (20.6)

Values are given as medians (range) or as number (%)

The following PCR mix and conditions were used. PCR mix per sample contained 15 mkl of 2x PCR Master Mix (Fermentas Life Sciences, Lithuania), forward and reverse primers (1 mM) and 28 ng of dried genomic DNA. The cycling conditions for PCR were: 5 minutes of initial denaturation at 95 °C, followed by 32 cycles of 15 seconds at 95 °C, 30 seconds at 56 °C, 30 seconds at 72 °C, and final extension for 10 minutes at 72 °C.

Statistical analysis. The chi-squared test was used for the calculation of the deviation from the Hardy-Weinberg equilibrium. Fisher's exact test and chi-square tests was used to examine allele/genotype association with presence of RA and other qualitative variables (positive RF, anti CCP and others). Dichotomised variables were created for RF and anti- CCP levels (for RF positivity 14 IU/ml; for antiCCP positivity > 5.0 units/ml). ANOVA tests was used to compare quantitative variables (age at onset of RA, VAS, DAS28, SW28, TEN28, CRP, ESR) between genotype-stratified subgroups of RA patients. A *P* value less than 0.05 was considered statistically significant. Odds ratios (OR) were calculated with a 95% confidence interval (CI). All statistics were conducted using the software package SPSS, version 13.0.

RESULTS

All genotyping results fit Hardy-Weinberg equilibrium. The distribution of alleles and genotypes is shown in Table 2.

There were no significant differences in the distribution of the *IL-18* gene alleles and genotypes between RA patients and the control group, but we found a difference in the frequencies of IL-18-607C/A and -656G/T genotypes, not alleles, between RA patients and the control group in women (Table 3).

We found a significant difference between frequencies of heterozygous versus homozygous genotypes; heterozygous genotypes -607CA and -656GT occurred more frequently in the RA group than in the control (Table 4). This difference was even larger when only women were considered (Table 5).

There were no differences in marker distributions between RA patients and the control for different age groups, men and control groups (data not shown). There were no differences found between age of onset RA, disease activity parameters (ESR, CRP, DAS28, SW28, TEN28), antibody status (RF positive/negative; antiCCP positive/negative), and RTG stages in RA group (data not shown).

DISCUSSION

In the present study we examined SNPs at the -607 and -656 positions of the *IL-18* gene in the patients with RA, in relation to disease susceptibility, activity and severity.

We found a significant difference in between RA patients and the control group in the distribution of heterozygous versus homozygous genotypes. A significant difference was also found when only the respective women groups were examined. Reports evaluating the role of *IL-18* promoter

Table 2

ALLELIC AND GENOTYPIC FREQUENCIES OF *IL-18* GENE POLYMORPHISMS IN LATVIAN PATIENTS WITH RA AND CONTROLS

	RA, n (%)	Controls, n (%)	*P*-value
IL-18-656G/T			
Genotypes	n = 104	n = 237	
GG/GT/TT	37 (35.6)/58 (55.8)/9 (8.7)	99 (41.8)/105 (44.3)/33 (13.9)	0.116
Alleles	2n = 208	2n = 474	
G/T	132 (63.5)/76 (36.5)	303 (63.9)/171 (36.1)	0.931*
IL-18-607C/A			
Genotypes	n = 104	n = 237	
CC/CA/AA	37 (35.6)/58 (55.8)/9 (8.7)	100 (42.2)/104 (43.9)/33 (13.9)	0.102
Alleles	2n = 208	2n = 474	
C/A	132 (63.5)/76 (36.5)	304 (64.1)/170 (35.9)	0.863**

Values are given as number (%)

Odds ratio and the confidence interval at the 95% confidence level is indicated as OR and 95%CI, respectively

*OR, 95%CI 0.986, 0.78–1.25

**OR, 95%CI 1.02, 0.81–1.29

Table 3

ALLELIC AND GENOTYPIC FREQUENCIES OF *IL-18* GENE POLYMORPHISMS IN LATVIAN WOMEN WITH RA AND CONTROLS

	RA, n (%)	Controls, n (%)	*P*-value
IL-18-656G/T			
Genotypes	n = 89	n = 191	
GG/GT/TT	31 (34.8)/51 (57.3)/7 (7.9)	79 (41.4)/85 (44.5)/27 (14.1)	0.097
Alleles	2n = 178	2n = 382	
G/T	113 (63.5)/65 (36.5)	243 (63.6)/139 (36.4)	1.000*
IL-18-607C/A			
Genotypes	n = 89	n = 191	
CC/CA/AA	31 (34.8)/51 (57.3)/7 (7.9)	80 (41.9)/84 (44.0)/27 (14.1)	0.084
Alleles	2n = 178	2n = 382	
C/A	113 (63.5)/65 (36.5)	244 (63.9)/138 (36.1)	0.925**

Values are given as number (%)

Odds ratio and the confidence interval at the 95% confidence level is indicated as OR and 95%CI, respectively

*OR, 95%CI 1.00, 0.74–1.28

**OR, 95%CI 1.01, 0.79–1.30

Table 4

GENOTYPIC FREQUENCIES OF *IL-18* GENE POLYMORPHISMS IN LATVIAN PATIENTS WITH RA AND CONTROLS

Genotypes	RA, n (%)	Controls, n (%)	*P*-value (χ^2)	OR, 95%CI
IL-18-656G/T				
GTvs.GG+TT	58 (55.8)/46 (44.2)	105 (44.3)/132 (55.7)	0.060	1.37, 1 00–1.90
GTvs.TT	58 (86.6)/9 (13.4)	105 (76.1)/33 (23.9)	0.098	1.66, 0.90–3.07
GT+TTvs.GG	67 (64.4)/37 (35.6)	138 (58.2)/99 (41.8)	0.337	1.20, 0.86–1.68
IL-18-607C/A				
CAvs.CC+AA	58 (55.8)/46 (44.2)	104 (43.9)/133 (56.1)	0.046	1.39, 1.01–1.93
CAvs.AA	58 (86.6)/9 (13.4)	104 (75.9)/33 (24.1)	0.097	1.67, 0.90–3.09
CA+AAvs.CC	67 (64.4)/37 (35.6)	137 (57.8)/100 (42.2)	0.281	1.23, 0.87–1.71

Values are given as number (%)

Odds ratio and the confidence interval at the 95% confidence level is indicated as OR and 95%CI, respectively

Table 5

GENOTYPIC FREQUENCIES OF *IL-18* GENE POLYMORPHISMS IN LATVIAN WOMEN WITH RA AND CONTROLS

Genotypes	RA, n (%)	Controls, n (%)	*P*-value (χ^2)	OR, 95%CI
IL-18-656G/T				
GT vs. GG+TT	51 (57.3)/38 (42.7)	85 (44.5)/106 (55.5)	0.054	1.42, 1.00–2.01
GT vs. TT	51 (87.9)/7 (12.1)	85 (75.9)/27 (24.1)	0.071	1.82, 0.91–3.65
GT+TT vs. GG	58 (65.2)/31 (34.8)	112 (58.6)/79 (41.4)	0.358	1.21, 0.84–1.74
IL-18-607C/A				
CA vs. CC+AA	51 (57.3)/38 (42.7)	84 (44.0)/107 (56.0)	0.041	1.44, 1.02–2.04
CA vs. AA	51 (87.9)/7 (12.1)	84 (75.7)/27 (24.3)	0.070	1.84, 0.92–3.68
CA+AA vs. CC	58 (65.2)/31 (34.8)	111 (58.1)/80 (41.9)	0.295	1.23, 0.85–1.77

Values are given as number (%)

Odds ratio and the confidence interval at the 95% confidence level is indicated as OR and 95%CI, respectively

polymorphisms in RA patients have shown variable results for different SNPs. Sivalingam *et al* (2003) found that controls had significantly higher frequency of the AA genotype at position -607 when compared to RA patients and concluded that the AA genotype at position -607 is associated with a protective effect against development of RA in Chinese individuals. Rueda *et al.* (2005) observed no statistically significant differences between RA patients and controls in allelic and genotypic frequencies of -607C/A and -137C/G IL-18 promoter polymorphisms. In addition, no association was found with the haplotypes inferred by the two polymorphisms and RA susceptibility. RA patients were stratified according to sex, age at the onset of the disease rheumatoid factor status, and extraarticular manifestations and no association was found with these polymorphisms. Therefore, the conclusion is that studied polymorphisms within the IL-18 promoter region do not play a major role in RA predisposition.

Gracie *et al.* (2005) found that the -607C/-137C haplotype was more prevalent in Caucasian RA patients than in a control group and concluded that SNP of both positions contribute to the genetic background of RA pathogenesis.

Lee *et al.* (2007) reported a significantly higher frequency of the 105A allele of the IL-18-105A/C SNP in Chinese rheumatoid arthritis patients compared with controls. The relative risk of rheumatoid arthritis was stronger in 105A homozygotes. Sugiura *et al.* (2006) showed that 12 SNP within the promoter region of the *IL-18* gene were associated with susceptibility to juvenile idiopathic arthritis (JIA) in Japanese patients. There was a strong association between the diplotype configuration of S01/S01 of the *IL-18* gene and JIA. T at position -656, A at position -607, and G at position -137 were the components of haplotype S01.

In the recent study by Pawlik *et al.* (2009), where seven *IL-18* gene SNPs were studied, no significant differences were found in the distributions of the genotypes except for rs360722 and haplotypes between RA patients and a control group. Age at RA diagnosis was lower in carriers of the -607 CC and rs187238 GG genotypes. Erosive disease was diagnosed more frequently in patients with the -607 CC and AC genotypes than in AA homozygotes.

A possible explanation for the discordant results could be linkage with other polymorphisms within the gene or within other genes; another one might be the variation of genetic susceptibility between ethnic groups (Burchard *et al.*, 2003). Allelic heterogeneity exists between ethnic groups, and different variations within the same gene can contribute to disease risk (Colhoun *et al.*, 2003).

It is well known that gene promoter polymorphisms can affect the level of protein production. The gene sequence can be associated with a cytokine phenotype. Homozygotes for the high-producer allele are the highest producers of the cytokine, homozygotes for the low-producer allele are the lowest producers of the cytokine and heterozygotes are intermediate. On the other hand, we can assume that homozygotes for the allele are the lowest producers of the cytokine and heterozygotes are the highest producers of the cytokine. Khripko *et al.* (2008) studied distribution of *IL-18* allele variants at positions -607C/A and -137G/C in healthy donors from Siberia and the influence of these allele variants on the level of IL-18 production by peripheral blood mononuclear cells (PBMCs). The authors found that lipopolysaccharide (LPS)-stimulated production of IL-18 by PBMC from healthy donors was significantly greater in

those carrying the CA genotype at the -607 position, compared with donors with the -607CC genotype.

RA is one of many autoimmune diseases that is predominant in women, as it has a female-to-male ratio 2:1 to 3:1. Sex hormones can have significant effects on the cells known to participate in RA. However, the specific mechanisms responsible for increased susceptibility to RA in women are uncertain. Padyukov *et al.* (2004) showed an association of IL10 -1087 genotypes in woman with RA compared with men and controls. The observed difference in frequencies of alleles and genotypes of the *IL-18* gene in positions -607 and -656 between RA patients and the control group in women suggests that RA in woman is a different disease subset. Further studies are needed and using a larger patient group, covering all rheumatology departments in Latvia. Patients should be enrolled based on the same inclusion criteria and with similar phenotypic data as used in the present study.

In conclusion, heterozygous states CA and GT at positions -607 and -656, respectively, of *IL-18* gene may influence susceptibility to RA. More studies on a larger patient group is needed, to explore this association in Latvian population. On the basis of the investigated *IL-18* polymorphisms, female patients with RA seem to represent a separate disease subgroup.

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INTERLEIKĪNA 18 GĒNA PROMOTERA POLIMORFISMI LATVIJAS SLIMNIEKIEM AR REIMATOĪDO ARTRĪTU

Interleikīns 18 (IL-18) ir proinflamatorais citokīns, kas iesaistīts reimatoīdā artrīta (RA) patoģenēzē. Dati par to, vai šī interleikīna promotera polimorfismi var būt neatkarīgi RA ģenētiskie marķieri, ir pretrunīgi. Šī pētījuma mērķis bija noteikt, vai *IL-18* gēna promotera polimorfismi -607 (rs 1946519) un -656 (rs 1946518) pozīcijā ir saistīti ar RA un tā īpatnībām Latvijas populācijā. Mēs izmeklējām 105 pacientus ar diagnosticētu RA saskaņā ar Amerikas reimatoloģijas koledžas diagnostiskajiem kritērijiem. Veselo kontroļu DNS un fenotipiskie dati iegūti no (Latvijas) Valsts iedzīvotāju genoma datu bāzes. Genotipi noteikti ar tiešu sekvenēšanu. Polimorfismi pētīti un alēļu un genotipu biežumi salīdzināti starp RA slimniekiem un kontrolgrupu. *P* vērtība mazāka par 0,05 pieņemta par statistiski būtisku. Mūsu pētījumā neatradām būtiskas atšķirības starp alēļu un genotipu biežumu starp RA slimniekiem un kontrolgrupu. Tendence uz asociāciju konstatēta starp IL-18-607C/A un -656G/T genotipiem sievietēm un kontrolgrupu (P = 0,084 un 0,097). Heterozigotiskus genotipus -607CA un -656GT novēroja biežāk RA grupā, nekā kontrolgrupā (P = 0,046, P = 0,060). Atšķirības novēroja, salīdzinot heterozigotisku genotipu -607CA un -656GT arī sievietēm (P = 0,041, P = 0,054). Secinājumi: heterozigotiskie genotipi -607CA un -656GT IL18 gēna promoterā var ietekmēt RA uzņēmību. Pamatojoties uz izmeklētiem IL18 polimorfismiem, RA slimnieces, šķiet, pārstāv atsevišķu slimības apakšgrupu.