Genetic Polymorphisms of *Foxp3* in Patients with Rheumatoid Arthritis

Agnieszka Paradowska-Gorycka, Monika Jurkowska, Anna Felis-Giemza, Katarzyna Romanowska-Próchnicka, Malgorzata Manczak, Slawomir Maslinski, and Marzena Olesinska

ABSTRACT. ***Objective.*** The aim of the study was to identify 2 polymorphic variants in the promoter region of the *Foxp3* gene and their possible association with susceptibility to and severity of rheumatoid arthritis (RA). The association between genetic factors and pathogenesis suggests that T cells take part in the induction of RA. The CD4+CD25highFoxp3+ subset of regulatory T cells plays an essential role in preventing autoimmunity and maintaining immune homeostasis.

Methods. Patients with RA (n = 274) and healthy individuals (n = 295) were examined for –3279 C/A and –924 A/G *Foxp3* gene polymorphisms by the polymerase chain reaction–restriction fragment-length polymorphism method. Serum Foxp3 levels in patients with RA and controls were measured with ELISA.

Results. *Foxp3* –3279 A and –924 G alleles were associated with significantly elevated risk of RA in the population tested ($p = 0.003$ and $p = 0.004$, respectively) compared to the wild-type alleles. Overall, –3279 C/A and –924 A/G *Foxp3* gene polymorphisms were in indistinct linkage disequilibrium with $D' = 0.481$ and $r^2 = 0.225$. From 4 possible haplotypes, frequencies of 2 (AG and CA) showed significant differences between both examined groups (respectively, $p < 0.001$ and $p = 0.007$). After appropriate adjustment of Bonferroni correction for multiple testing, the genotype-phenotype analysis showed no significant correlation of the *Foxp3* –3279 C/A and –924 A/G polymorphisms with the disease activity, joint damage, laboratory variables, and extraarticular manifestation in patients with RA. Serum Foxp3 level was significantly higher in patients than in controls ($p < 0.0001$).

Conclusion. Current findings indicated that the *Foxp3* genetic polymorphism and the Foxp3 protein level may be associated with susceptibility to RA in the Polish population. (J Rheumatol First Release Dec 1 2014; doi:10.3899/jrheum.131381)

Key Indexing Terms:
FOXP3 GENE POLYMORPHISMS SERUM LEVELS RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is one of the most common chronic autoimmune inflammatory diseases, where environmental, genetic, and immune factors contribute to development and progression of its inflammatory manifestations. In RA, congenital or acquired defects of the synovial membrane barrier may impair immune tolerance mechanisms. Inadequate immune response may result in overactivated T cells, B cells, macrophages, neutrophils, and synovial fibroblasts, as well as in synthesis of proinflammatory mediators that contribute to the damage of cartilage and joints[1,2,3,4]. The association between genetic factors (predominantly HLA haplotypes) and pathogenesis of RA suggests that antigen-specific T cells, especially CD4+ Th cells, take part in the induction of the disease[5]. Unlike the significance of proinflammatory mediators (such as tumor necrosis factor-α), which has been well documented in the pathogenesis of RA, the relevance of T cells in the disease is not clear and requires further elucidation[6].

Naive Th cells differentiate into Th1, Th2, Th17, and regulatory T cell (Treg) subtypes, which have different functional properties[7]. Treg cells, originally described as CD4+CD25+ regulatory T cells, play an essential role in preventing autoimmunity and maintaining immune homeostasis, as well as controlling T cell responses[8,9]. However, the precise mechanisms underlying suppression mediated by Tregs are still poorly understood. In recent years, increasing attention has been focused on the role of regulatory T cells

From the departments of Biochemistry and Molecular Biology, Connective Tissue Diseases, and Epidemiology and Health Promotion, Institute of Rheumatology; the Department of Pathophysiology, Medical University of Warsaw, Warsaw, Poland.

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A. Paradowska-Gorycka, PhD; M. Jurkowska, PhD, Department of Biochemistry and Molecular Biology, Institute of Rheumatology; A. Felis-Giemza, MD; K. Romanowska-Próchnicka, MD, Department of Connective Tissue Diseases, Institute of Rheumatology; M. Manczak, MS, Department of Epidemiology and Health Promotion, Institute of Rheumatology; S. Maslinski, Professor, Department of Pathophysiology, Medical University of Warsaw; M. Olesinska, Professor, Department of Connective Tissue Diseases, Institute of Rheumatology.

Address correspondence to Dr. A. Paradowska-Gorycka, Department of Biochemistry and Molecular Biology, Institute of Rheumatology, Spartańska 1, 02-637 Warsaw, Poland. E-mail: paradowska_aga@interia.pl

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in RA and there is emerging evidence that these cells may indeed function as important regulators of immune response in RA. Treg function during the control of cell homeostasis can determine whether an autoimmune disease will develop. Further, in the chronic inflammatory process, an increased number of Treg cells may even be harmful, through the inhibition of the natural course of an effective immune response, and in consequence conversion of a physiological inflammatory process into a chronic autoimmune inflammation[10,11].

Although Treg cells express numerous markers, forkhead box P3 (Foxp3) is the unique Treg-specific marker, important in the development of "naturally occurring" Tregs in the thymus (nTreg). It is also necessary to maintain the suppressive function of "induced" Treg (iTreg) cells in peripheral tissues[12,13,14]. Foxp3, encoded by the human *Foxp3* gene located on chromosome Xp11.23[15], belongs to the forkhead/winged helix box-containing transcription factor family with a unique forkhead domain. This domain is critical for nuclear localization and DNA binding, which lead to transcriptional activation and/or repression[13,16,17,18]. Foxp3 acts as a transcriptional activator for genes that are typically upregulated in nTregs, while it represses transcription of both Th1 and Th2 cytokines[19]. The acquisition of the nTreg phenotype appears to require high and persistent expression of Foxp3 to stabilize and amplify a Treg genetic program[20,21]. Moreover, polymorphisms within the *Foxp3* locus are known to alter Foxp3 expression and function, suggesting that they may play a role in the initiation or maintenance of Treg-dependent chronic inflammatory states[20,22,23,24,25,26]. To test this hypothesis, we examined, to our knowledge for the first time, 2 candidate single-nucleotide polymorphisms (SNP) in the *Foxp3* gene promoter region, –3279 C/A (rs3761548) and –924 A/G (rs2232365), and explored their association with susceptibility to and severity of RA in the Polish population.

MATERIALS AND METHODS

Study population. The study group consisted of 274 patients with RA (256 women, 93%) and of 295 healthy individuals (87% women vs 13% men, age 18–65 yrs) without history of immunological diseases. All patients fulfilled the American College of Rheumatology (ACR 1987) criteria for RA. Patients with RA were recruited from the Connective Tissue Diseases Department of the Institute of Rheumatology in Warsaw. All patients signed an informed consent, and clinical data were collected from patient files and questionnaires. The clinical and biochemical characteristics of patients included into the study are presented in Table 1. Our study was approved by the Research Ethics Committee of the Institute of Rheumatology in Warsaw.

DNA extraction. Genomic DNA was extracted from whole blood collected in EDTA tubes from patients with RA and the control group using the standard isothiocyanate guanidine extraction method and/or the QIAamp DNA Blood Mini Kit (Qiagen). DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm.

Genotyping. Two candidate SNP in the *Foxp3* gene at position –3279 C/A and –924 A/G were determined using the polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) method. Amplification reaction was performed with 200 ng of genomic DNA in a 50-μl PCR mixture using 10 pmol of each primer, 0.25 mM each dNTP (Qiagen), 1U HotStar Taq Polymerase (Qiagen), and 1× PCR buffer (containing 1.5 μM magnesium chloride, Qiagen).

Table 1. Clinical and biochemical characteristics of patients with rheumatoid arthritis (RA).

Characteristics	Patients with RA	
	N	Median (IQR)
Age, yrs	268	56 (50–64)
Disease duration, yrs	265	10 (6–17)
Larsen	271	3 (3–4)
No. swollen joints	266	3 (1–7)
No. tender joints	266	8 (4–13)
ESR, mm/h	270	28 (15–42)
CRP, mg/l	269	12 (5–27)
Hemoglobin, g/dl	269	12.7 (11.6–13.5)
VAS, mm	266	50 (30–67)
DAS28-CRP	265	5.0 (3.8–5.9)
HAQ	243	1.5 (0.9–2.0)
PLT, $\times 10^3$/mm^3	268	308 (251–379)
Creatinine	269	0.7 (0.6–0.8)
	N	n (%)
RF presence	271	171(63)
Anti-CCP presence	270	210 (78)
Morning stiffness	243	216 (89)
Organ symptoms	270	75 (28)
Coronary artery disease	266	28 (11)
Hypertension	268	110 (41)
Myocarditis	265	7 (3)
Diabetes	266	14 (5)
Renal syndrome	266	2 (1)
Renal failure	266	12 (5)

N: no. patients with clinical information; n: no. patients with positive clinical manifestation; IQR: interquartile range; DAS28: Disease Activity Score for 28 joints; VAS: visual analog scale (range 0–100); HAQ: Health Assessment Questionnaire (range 0–3); CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; PLT: platelet; RF: rheumatoid factor (> 34 IU/ml); anti-CCP: anticyclic citrullinated peptide antibodies (> 17 U/ml).

Foxp3 *–3279 C/A (rs 3761548)*. Primer sequences were forward (5′ – CTT AAC CAG ACA GCG TAG AAG G-3′) and reverse (5′ – CAT CAT CAC CAC GCT CTG G-3′). The method for PCR included an initial denaturing at 95°C for 15 min, followed by 35 cycles at 94°C for 45 s; 55.6°C for 45 s; and 72°C for 1 min with a final extension at 72°C for 10 min. Ten μl of PCR product (399 bp in length) was digested with 1 μl PstI (Fermentas) at 37°C for 30 min, separated on a 2.5% agarose gel and viewed with ethidium bromide staining under ultraviolet light. PstI digestion of the PCR product yielded 399 bp for the undigested allele A, and 188 and 211 bp for allele C (Figure 1A).

Foxp3 *-924 A/G (rs2232365)*. Primer sequences were forward: 5′ – AGG AGA AGG AGT GGG CAT TT -3′; reverse: 5′ – TGT GAG TGG AGG AGC TGA GG-3′. The protocol for the PCR was as follows: 95°C for 5 min and 35 cycles of denaturing at 95ºC for 45 s, annealing at 55°C for 45 s and extension at 72ºC for 1 min and a single final extension at 72ºC for 10 min. Ten μl of PCR product (249 bp) was digested with 1 μl BsmBI (Fermentas) at 37°C for 15 min and separated on a 3% agarose gel and viewed with ethidium bromide staining under ultraviolet light. BsmBI digestion of PCR products yielded 249 bp for the A allele, whereas for allele G, 117 and 132 bp fragments were observed (Figure 1B).

To confirm the accuracy of the method, randomly selected patients

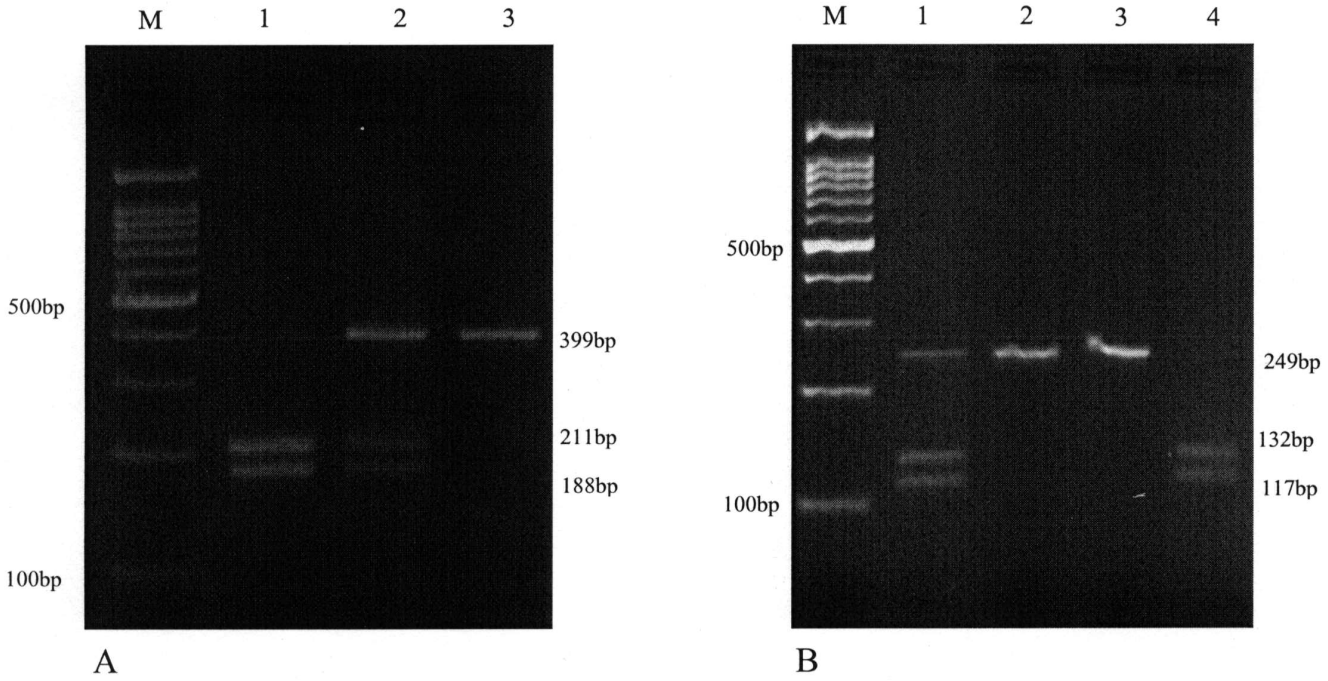

Figure 1. Identification of the Foxp3 –3279 A/C and –924 A/G genotypes. A. Foxp3 –3279 A/C; M is the 100 base pair (bp) marker. Lane 1 is homozygous for the wild-type allele (AA); lane 2 is heterozygous (AC); and lane 3 is homozygous for the polymorphic allele (CC). B. Foxp3 –924 A/G; M is the 100 bp marker. Lane 1 is heterozygous (AG); lane 2 and 3 are homozygous for the wild-type allele (AA); and lane 4 is homozygous for the polymorphic allele (GG).

were analyzed by direct sequencing, using an ABI PRISM Sequencer (Applied Biosystems).

Detection of Foxp3 levels. For quantitative determination of Foxp3 serum levels, samples from patients and healthy subjects were separated from peripheral venous blood at room temperature and stored at –86°C until analysis. The levels of circulating Foxp3 in serum were determined using commercially available ELISA kits (Uscn Life Science), according to the manufacturer's instructions. The minimum level of detection for Foxp3 was 0.121 ng/ml. Each sample was assayed in duplicate and the intraassay coefficient of variation was < 10%. The developed color reaction was measured at OD450 units on an ELISA reader (El × 800, BIO-TEK Instruments).

Statistical analysis. The associations between the genotypes of the Foxp3 –3279 C/A and –924A/G and 4730 T/C polymorphisms and risk of RA were estimated by computing OR and 95% CI. Statistical significance for the 2 SNP comparison was set at $p < 0.025$ (according to Bonferroni correction). The association between target SNP and the risk of RA was analyzed by unconditional logistic regression under 4 genetic models including codominant, dominant, recessive, and overdominant models. For genetic association analyses, all polymorphisms were tested for deviations from the Hardy-Weinberg Equilibrium (HWE) using the HardyWeinberg Simulator software. Linkage disequilibrium (LD) and coefficient (D′ and r^2) for haplotypes and their frequencies were performed using the genetic statistical software SHEsis (http://analysis.bio-x.cn)[27,28]. The association between SNP and clinical/serological variables was assessed by the chi-squared test with Yates correction (categorical variables) or the Mann-Whitney U test (continuous variables). We used Bonferroni correction to adjust p values for multiple measures. Bonferroni-corrected α-level of $p < 0.003$ (0.05/16) was considered statistically significant.

RESULTS

Frequency distribution of the Foxp3 gene variants in patients with RA and controls. The distributions of genotype and allele frequencies of the polymorphisms –3279 C/A and –924 A/G in *Foxp3* among patients and controls, as well as their associations with the risk of RA are shown in Table 2. Four genetic models, including codominant, dominant, recessive, and overdominant, were applied to assess the association of SNP within the *Foxp3* gene and RA risk.

The analysis of polymorphism –3279 C/A frequencies revealed significant differences in the case-control distribution. Under the codominant model, the frequency of the CA and AA genotypes was significantly higher in patients with RA compared to the healthy subjects ($p = 0.0004$ and $p = 0.037$). Similarly, under the dominant model (CA + AA vs CC) the association was also significant (OR 1.8; CI 1.3–2.6; $p = 0.0004$). Under the overdominant model, the frequency of the CA genotype was significantly higher (47% vs 35%; $p = 0.003$) and that of the CC + AA genotypes was lower (53% vs 65%) in patients with RA compared to the healthy subjects. Under the recessive model (CC + CA vs AA), genotype distributions were not statistically significant (OR 1.2; CI 0.8–1.8); $p = 0.437$.

Regarding the –924 A/G *Foxp3* gene polymorphism, patients with RA showed genotype and allele distributions significantly different from the control subjects. Under the codominant model, the genotype frequency comparison (AA vs AG) was statistically significant (OR 2.3; CI 1.6–3.4; $p = 0.00001$). Under the dominant model (AA vs AG + GG), the association was also significant (OR 2.8; CI

Table 2. Distribution of genotypes and allele frequencies of Foxp3 SNP among Polish patients with rheumatoid arthritis (RA) and healthy subjects.

Foxp3 –3279 C/A Model	Genotype	RA, n (%)	Controls, n (%)	OR (95% CI)	p*
Codominant					
	CC	92 (34)	143 (49)	1	—
	CA	129 (47)	104 (35)	1.9 (1.3–2.8)	**0.0004**
	AA	51 (19)	48 (16)	1.7 (1.03–2.65)	**0.037**
Dominant					
	CC	92 (34)	143 (48)	1	—
	CA + AA	180 (66)	152 (52)	1.8 (1.3–2.6)	**0.0004**
Recessive					
	CC + CA	221 (81)	247 (84)	1	—
	AA	51 (19)	48 (16)	1.2 (0.8–1.8)	0.437
Overdominant					
	CC + AA	143 (53)	191 (65)	1	—
	CA	129 (47)	104 (35)	1.7 (1.2–2.3)	**0.003**
	Alleles				
	C	313 (58)	390 (66)	1	—
	A	231 (42)	200 (34)	1.4 (1.1–1.8)	**0.003**
Foxp3 –924 A/G Model	Genotype				
Codominant					
	AA	57 (21)	111 (38)	1	—
	AG	198 (73)	166 (56)	2.3 (1.6–3.4)	**0.00001**
	GG	17 (6)	18 (6)	1.8 (0.9–3.8)	0.102
Dominant					
	AA	57 (21)	111 (38)	1	—
	AG + GG	215 (79)	184 (62)	2.8 (1.6–3.3)	**0.00001**
Recessive					
	AA + AG	255 (94)	277 (94)	1	—
	GG	17 (6)	18 (6)	1.03 (0.52–2.03)	0.942
Overdominant					
	AA + GG	74 (27)	129 (44)	1	—
	AG	198 (73)	166 (56)	2.1 (1.5–3.0)	**0.00004**
	Alleles				
	A	312 (57)	388 (66)	1	—
	G	232 (43)	202 (34)	1.4 (1.1–1.8)	**0.0036**

*Chi-squared test, $p \leq 0.025$ was considered significant. P values in bold face are significant. SNP: single-nucleotide polymorphisms.

1.6–3.3; p = 0.00001). Similarly, under the overdominant model (AA + GG vs AG), we obtained significant results (OR 2.1; CI 1.5–3.0; p = 0.00004).

The statistical analysis revealed that the *Foxp3* gene –3279 A allele and the –924 G allele were associated with significantly increased risk of RA in the Polish population (p = 0.003 and p = 0.004) compared with the wild-type –3279 C and –924 A alleles.

To check for deviation in the distribution of *Foxp3* –3279 C/A and –924 A/G genotypes, we tested for deviation from HWE, by comparing observed-to-expected distributions in both patients and control groups. The genotypes in patients with RA for –3279 C/A were in equilibrium, whereas the genotypes in controls for –3279 C/A (p = 0.03) and both patients (p < 0.01) and controls (p = 0.01) for –924 A/G polymorphisms showed departures. Genotyping (PCR-RFLP and sequencing) was repeated on randomly selected samples, giving complete conformity of the result.

Foxp3 *haplotypes and risk of RA*. In this study, *Foxp3* haplotypes were assessed for the RA group and the control group. Analysis by the SHEsis program demonstrated that –3279 C/A and –924 A/G *Foxp3* gene polymorphisms were in indistinct LD with $D' = 0.481$ and $r^2 = 0.225$. Still, both SNP were assessed in haplotype analysis. The 4 potential haplotypes were formed, as presented in Table 3. There was a predominance of AG haplotype in the cases and CA haplotype in the controls. Haplotype AG indicated significantly higher risk for RA (OR 1.581; 95% CI 1.211~2.066; p < 0.001). Other haplotypes, AA and CG, showed no significant difference.

The association of the Foxp3 *–3279 C/A and –924 A/G polymorphisms with clinical characteristics of patients with RA*. Because we found that the examined *Foxp3* variants may be associated with susceptibility to RA, we next analyzed the potential association between both the *Foxp3* gene SNP and laboratory and disease activity variables in our RA group. Without Bonferroni correction, the genotype-phenotype analysis showed significant correlation of the *Foxp3* –3279 C/A polymorphism with the Larsen score,

Table 3. *Foxp3* haplotypes in patients with rheumatoid arthritis (RA) and controls.

Haplotype	RA, 2n = 556 (%)	Controls, 2n = 590 (%)	p*	OR (95% CI)
AA	65 (11.7)	73.3773 (12.4)	0.714	0.936 (0.656~1.335)
AG	167.79168 (30.3)	126.63127 (21.5)	**< 0.001**	1.581 (1.211~2.066)
CA	252.79253 (45.5)	314.63315 (53.3)	**0.007**	0.730 (0.578~0.921)
CG	70 (12.6)	75.3775 (12.8)	0.9409	0.987 (0.697~1.398)

*Fisher's test. P values in bold face are considered significant.

visual analog scale (VAS), rheumatoid factor (RF) presence, and sex (data not shown). The Larsen score, as well as mean value of the VAS score, was significantly increased in RA patients with –3279 CA or –3279AA genotypes in comparison to patients with wild-type genotype (p = 0.0146 and p = 0.0369, respectively). In our study, the number of RA women with –3279 CA or –3279AA genotypes was higher than the number of RA women with –3279 CC (p = 0.0437). Moreover, in carriers of the polymorphic –3279 A allele, RF was present significantly less frequently than in patients with 2 wild-type alleles (p = 0.0346). However, after Bonferroni correction for multiple testing there was no significant association between SNP and RA phenotype (Table 4). No association could be detected between the *Foxp3* –3279 C/A variant and other disease activity and laboratory variables and extraarticular manifestation (ExRA) among patients with RA.

We did not find significant differences in allele frequencies and genotype distribution of the *Foxp3* –924 A/G polymorphism among patients divided according to disease activity and ExRA (data not shown).

Foxp3 *levels in patients/controls and in relation to RA clinical variables*. Samples were stratified into positive and negative based on detected levels of Foxp3 (> 0.121 ng/ml). As shown in Table 5 and Figure 2, the number of Foxp3-positive patients with RA was significantly higher than the number of positive healthy subjects (51% RA vs 18% controls). We next conducted a comparative analysis between positive and negative patients in relation to clinical variables and ExRA. However, association analysis did not show any significant relationship between the studied serum protein levels and clinical and biochemical variables in our patients with RA after Bonferroni correction (Table 6). Without correction, we found that among the Foxp3-positive patients, the mean value of the 28-joint Disease Activity Score–C-reactive protein (DAS28-CRP), mean value of creatinine, number of women, and organ symptoms were significantly lower in comparison to Foxp3–negative patients (p = 0.0456, p = 0.0206, p = 0.0088, and p = 0.0313, respectively; data not shown). In patients with Foxp3 concentration > 0.121 ng/ml, we observed a tendency toward a lower variable of the disease activity and joint

Table 4. Disease activity and laboratory variables in relation to *Foxp3* –3279 C/A.

Characteristic	CC		CA +AA		p*
	N	Median (IQR)	N	Median (IQR)	
Age, yrs	89	55 (51–63)	179	57 (49–65)	0.7265
Disease duration, yrs	88	11.5 (6.5–17)	177	10 (6–17)	0.4302
Larsen	92	3 (3–4)	179	3 (2–4)	0.0146
No. swollen joints	88	3 (1–7.5)	178	3 (1–6)	0.1218
No. tender joints	88	9 (4–14)	178	7 (3–13)	0.1218
ESR, mm/h	92	30 (15–44)	178	25 (14–41)	0.4058
CRP, mg/l	92	12 (6.6–28.1)	177	11 (5–25)	0.3688
Hemoglobin, g/dl	92	12.8 (11.7–13.5)	177	12.6 (11.5–13.5)	0.5487
VAS, mm	90	55.5 (32–70)	176	47.5 (30–65)	0.0396
DAS28-CRP	89	5.1 (3.8–6.0)	176	4.9 (3.8–5.7)	0.1231
HAQ	81	1.6 (1–2)	162	1.4 (0.9–1.9)	0.2431
PLT × 10^3/mm^3	91	308 (238–393)	177	308 (259–377)	0.7964
Creatinine	92	0.7 (0.6–0.8)	177	0.7 (0.6–0.8)	0.4552
	CC		CC + CA		p**
	N	n (%)	N	n (%)	
Women	92	82 (89)	180	172 (96)	0.0437
RF presence	92	66 (72)	179	105 (59)	0.0346
Anti-CCP presence	91	73 (91)	163	143 (88)	0.3184

*Mann-Whitney U test. ** Chi-square test with Yates' correction. IQR: interquartile range; DAS28: Disease Activity Score for 28 joints; VAS: visual analog scale (range 0–100); CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; PLT: platelet; RF: rheumatoid factor (> 34 IU/ml); anti-CCP: anticyclic citrullinated peptide antibodies (> 17 U/ml).

Table 5. Serum level of Foxp3 in patients with rheumatoid arthritis (RA) and healthy subjects.

Foxp3 Protein	RA, n (%)	Controls, n (%)	p*	OR	95% CI
Positive	138 (51)	54 (18)	**0.0001**	4.6	3.15–6.72
Negative	134 (49)	241 (82)		0.2	0.15–0.32

*Chi-squared test with Yates correction. P = RA vs controls; p ≤ 0.05 was considered significant and is given in bold face.

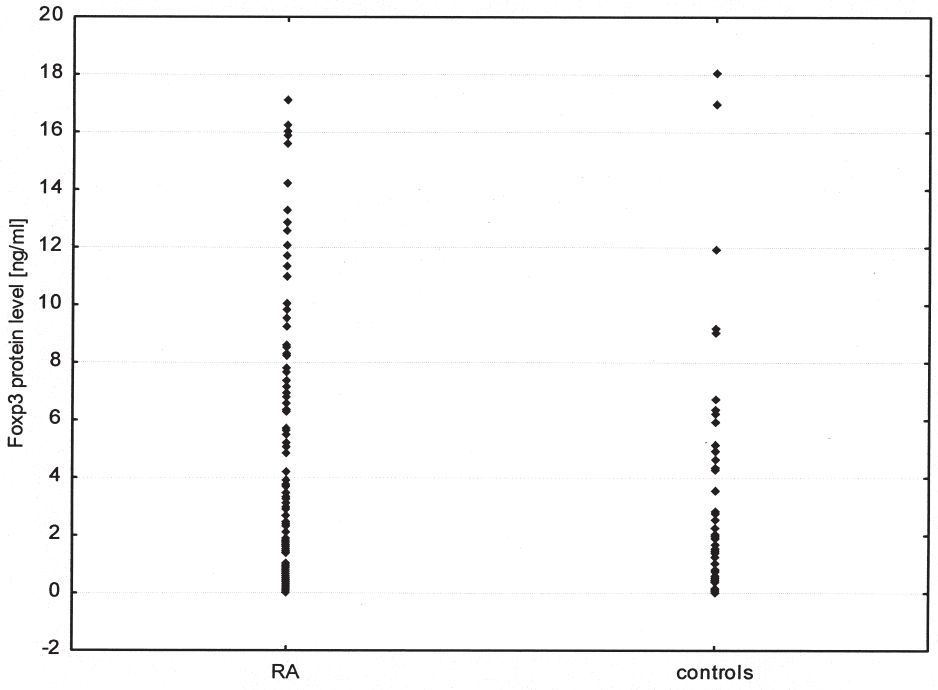

Figure 2. Variation in Foxp3 expression levels in patients with rheumatoid arthritis (RA) and control group.

damage than in patients with Foxp3 concentration < 0.121 ng/ml (Table 6).

Foxp3 *gene polymorphisms and serum Foxp3 levels*. To study the relationship between genotype and phenotype, we investigated Foxp3 serum levels in patients with RA and controls regarding *Foxp3* gene polymorphisms.

First, we examined the relationship between Foxp3 expression levels in Foxp3–positive/negative patients with RA and the Foxp3–positive/negative control group in relation to –3279 C/A and –924 A/G Foxp3 genotypes. In this case, we found no significant association, neither among patients with RA nor in healthy subjects (data not shown).

We next conducted a comparative analysis between patients with RA and control group according to –3279 C/A and –924 A/G *Foxp3* genotypes. Serum levels of Foxp3 in patients with RA who had –3279 AA, CA, and AA genotypes were significantly higher than in controls with the same –3279 C/A genotypes (p = 0.001, p = 0.001, p = 0.003; Figure 3). Increased serum levels of Foxp3 were also observed in patients with RA who had –924 AA and AG genotypes compared to controls with the same genotypes (p = 0.001 in both cases; Figure 4). Further, serum levels of Foxp3 among patients with RA who had the –924 GG genotype were not significantly different from those detected in the sera of healthy donors with –924 GG genotypes.

DISCUSSION

RA is a polyetiological disease whose genetic component comprises the cumulative effect of several common gene variants rather than mutations of any 1 particular gene[6]. Although many studies have shown that certain HLA-DR alleles bearing common sequences (called shared epitopes) play a significant role in genetically determined susceptibility to RA[29,30,31], their presence is not sufficient to induce the disease, so the total genetic background of RA remains unexplained. Other genes may play a role in the patho-

Table 6. Correlation of Foxp3 protein concentration with the various clinical characteristics in rheumatoid arthritis (RA).

Characteristic	Foxp-positive		Foxp-negative		p*
	N	Median (IQR)	N	Median (IQR)	
Age, yrs	135	55 (49–64)	133	57 (51–65)	0.3855
Disease duration, yrs	134	10 (6–17)	131	11 (7–17)	0.6046
Larsen	137	3 (3–4)	134	3 (2–4)	0.8591
No. swollen joints	136	2.5 (1–6)	130	3 (1–7)	0.2384
No. tender joints	136	7.5 (3–12.5)	130	8 (5–14)	0.3053
ESR, mm/h	136	25 (12–41)	134	29.5 (18–43)	0.1040
CRP, mg/l	135	10 (4–21)	134	12 (6.3–31)	0.0708
Hemoglobin, g/dl	136	12.6 (11.5–13.5)	133	12.9 (11.7–13.6)	0.3856
VAS, mm	134	49 (30–64)	132	52 (31.5–70)	0.1254
DAS28-CRP	134	4.7 (3.7–5.7)	131	5.1 (4.1–6.0)	0.0456
HAQ	119	1.5 (0.8–2)	124	1.5 (1–2)	0.2516
PLT × 10^3/mm^3	137	317 (259–386)	131	305 (244–362)	0.3168
Creatinine	136	0.7 (0.6–0.8)	133	0.7 (0.6–0.8)	0.0206
	Foxp-positive		Foxp-negative		p**
	N	n (%)	N	n (%)	
Women	138	123 (89)	134	131 (98)	0.0088
RF presence	137	79 (58)	134	92 (69)	0.0608
Anti-CCP presence	136	104 (77)	134	106 (79)	0.6027

* Mann-Whitney U test. ** chi-squared test with Yates correction. N: no. patients with clinical information; n: no. patients with positive clinical manifestation; IQR: interquartile range; DAS28: Disease Activity Score for 28 joints; VAS: visual analog scale (range 0–100); HAQ: Health Assessment Questionnaire (range 0–3); CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; PLT: platelet; RF: rheumatoid factor (> 34 IU / ml); anti-CCP: anticyclic citrullinated peptide antibodies (> 17 U/ml).

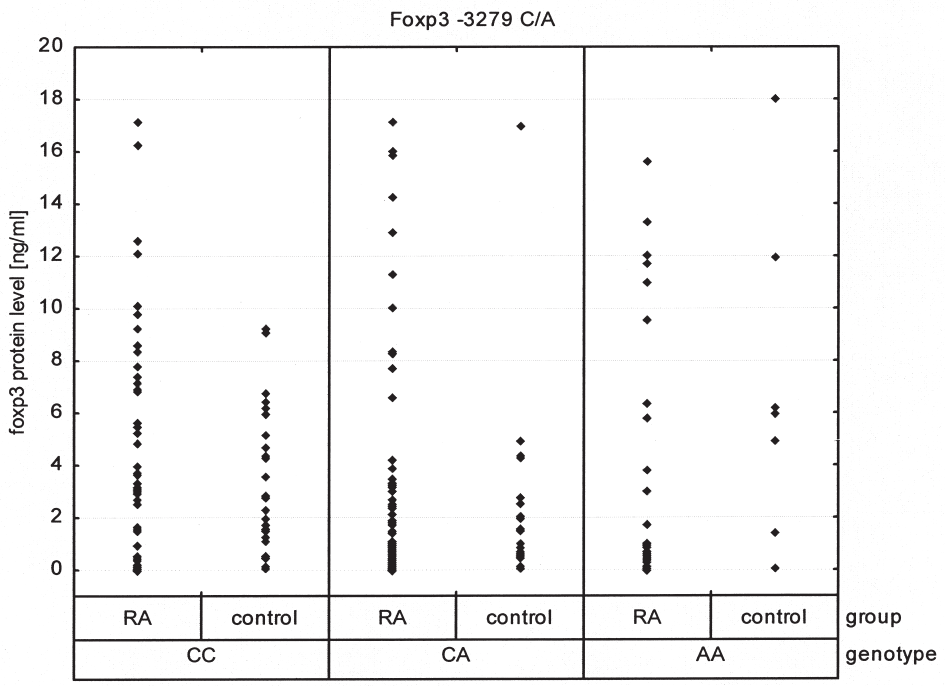

Figure 3. Variation in Foxp3 expression levels in patients with rheumatoid arthritis (RA) and control group in relation to –3279 C/A *Foxp3* genotypes. RA vs control group: CC genotype (p = 0.001); CA genotype (p = 0.001); AA genotype (p = 0.003).

genesis of RA (such as genes which, similarly to HLA, control detection of some bacterial and viral products). Analysis of polymorphisms within the *Foxp3* gene (which have not previously been widely used in the diagnosis of

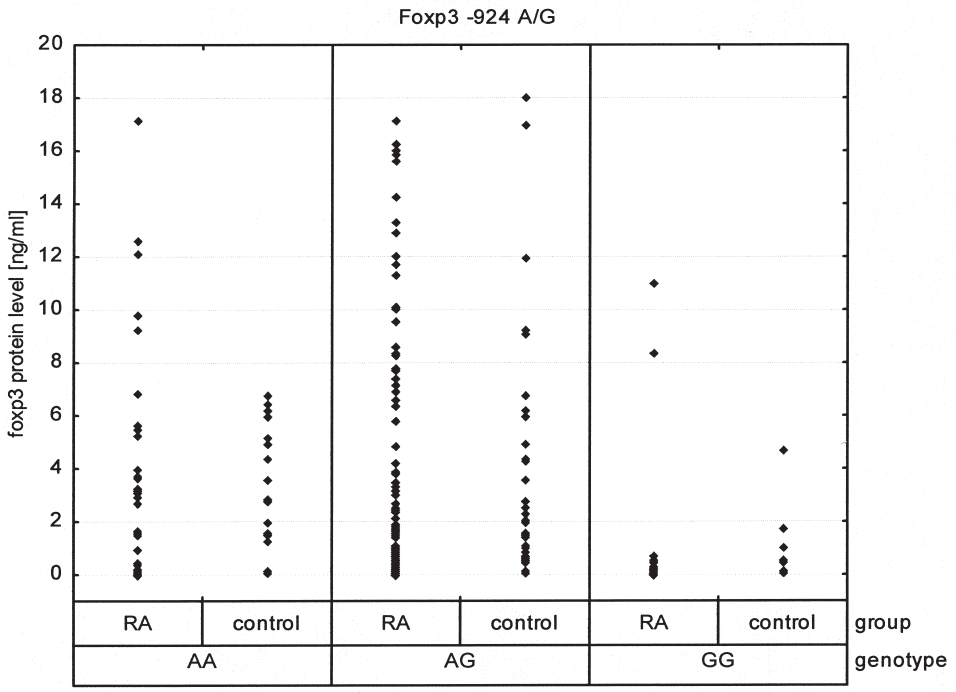

Figure 4. Variation in Foxp3 expression levels in patients with rheumatoid arthritis (RA) and control group in relation to –924 A/G *Foxp3* genotypes. RA vs control group: AA genotype (p = 0.001); AG genotype (p = 0.001); GG genotype (p = 0.586).

RA) may help to uncover their correlations with some biochemical and laboratory findings. It is known that *Foxp3* may be a candidate gene for RA, first because of its location at chromosome Xp11.2 (one of the susceptibility loci for autoimmune diseases[1,32,33,34,35,36]), and second through its important role in confirming and governing Treg cell action[24,37,38]. The precise quantitation of Foxp3 expression levels and Treg cell number and function in the autoimmune process is therefore of great importance for the understanding of the pathogenesis not only in RA, but also in other autoimmune disorders.

In our study, we observed that Foxp3 serum levels were significantly higher in patients with RA than in healthy subjects (Table 5), reflecting the ongoing inflammatory process in patients and attempts to keep it under control. Several previous reports have described the *Foxp3* mRNA[39,40], but our report is the first study, to our knowledge, exploring the protein expression in the peripheral blood of patients with RA. Because protein expression levels are often correlated with mRNA expression, we considered our results consistent with previous reports. While the p values presented here have not been corrected for multiple testing, we demonstrated that among the Foxp3-positive patients, the mean value of DAS28-CRP, the levels of CRP and platelet, as well as organ symptoms, were lower in comparison to Foxp3-negative patients. However, if we implemented the conservative Bonferroni correction, we observed that in patients with Foxp3 concentration > 0.121 ng/ml, there was a tendency toward lower variables of disease activity and joint damage than in patients with Foxp3 concentration < 0.121 ng/ml (Table 6). The differences were not statistically significant. Our results indicate that Foxp3-positive patients have lower disease activity and probably an increased number of Treg cells than Foxp3-negative patients and controls. Moreover, as shown in earlier studies, Treg cells isolated from patients with active RA had reduced expression of Foxp3 and a decreased ability to suppress the proliferation and secretion of cytokines, proliferation of T effector cells, expression of chemokine receptors, and cytolytic function, which also supports our observations[10,14,41,42,43]. Therefore, we conclude that the increased number of Treg cells and thus increased expression of Foxp3 in the serum of patients with RA may be a sign of antagonism between Th17 and Treg cells, shifting the equilibrium to the direction of Treg cells. Further, expression of Foxp3 is necessary for the anti-inflammatory capacity of Tregs. Because it is crucial to the modulation of active disease and inhibition of autoimmune responses, it can serve as a measure of disease activity[44].

Structural, quantitative, or regulatory polymorphisms at the *Foxp3* locus may lead to a lack of functional Treg cells and contribute to the susceptibility to some autoimmune conditions. The *Foxp3* polymorphisms, including both examined here, have been associated with susceptibility to

psoriasis, allergic rhinitis, autoimmune thyroid disease, and systemic lupus erythematosus[26,38,45,46], but not to systemic sclerosis, endometriosis, Crohn disease, or breast cancer[47,48,49]. Sanchez, *et al*[22], in the sole report exploring *Foxp3* polymorphisms in RA, studied the $(GT)_n$ microsatellite sequence in the promoter region of the *Foxp3* gene. They concluded that this SNP did not play a relevant role in the susceptibility to autoimmune diseases, including RA, in the Spanish population. Considering differences in genetic predispositions between populations and an important role of the Foxp3 in immune homeostasis, we decided to carry out an analysis of selected polymorphisms located in this gene in relation to RA. We chose the –3279 C/A and –924 A/G polymorphisms located in putative DNA-binding sites for transcriptional factor (TF) specificity protein 1 (Sp-1) and GATA-3, respectively[25,50]. These polymorphisms may potentially alter gene expression by changing the binding of the above TF and subsequently lead to defective transcription of *Foxp3*[8]. However, this needs to be verified in further studies.

Data from epidemiological studies showed a marked difference in the distribution of both analyzed polymorphisms among different ethnic groups. A comparison of our data with the HapMap database (International HapMap Project: http://hapmap.ncbi.nlm.nih.gov/) revealed that the *Foxp3* minor –3279 A allele frequency was higher in Polish subjects (34%) than in populations of Africa, Japan, and China (from 4% to 26%), but at the same time lower than in other European populations (41%). Discrepancies in genotype frequencies between reports may be explained by the heterogeneity of the analyzed diseases and distinct ethnicities as well as limited sample size. The results of our study also showed significant difference between patients with RA and controls in genotype distribution and allele frequencies for both examined polymorphisms (Table 2). The subjects carrying the –3279 A and –924 G variant alleles were at a higher risk for RA than those carrying the –3279 C and –924 A variant alleles, indicating that the –3279 A and –924 G variant alleles are putative RA risk alleles in the Polish population. However, in our study we observed that genotype distribution in control groups for –3279 C/A and in patients with RA and controls for –924 A/G *Foxp3* polymorphisms was not concordant with HWE. Deviations from HWE can be very informative. They could imply a sampling bias, mistyping of genotypes, inbreeding, differential survival of marker carriers, genetic drifting, ethnic differences, migration, population stratification, or a combination of these. Although deviation from the HWE often indicates a poor quality of genotyping, it can also be caused by small sample size.

Minimization of genotyping errors was achieved by repetition of testing and sequencing of randomly selected samples. However, in case subjects, deviation from HWE, assuming sources of error have been eliminated, may indicate the association of a locus with disease. The analysis of haplotype or gene interaction should also be considered. Consistent with this observation, we provide evidence for association of *Foxp3* –3279 A and –924 G haplotypes with the risk of RA, suggesting that the effect of the gene on disease risk may not be limited to a single SNP. To our knowledge this is the first haplotype-based association study showing the combined effect of the –3279 C/A and –924 A/G *Foxp3* polymorphisms.

We also investigated the influence of –3279 C/A and –924 A/G *Foxp3* gene polymorphisms on clinical and laboratory characteristics of our patients. Our detailed genotype-phenotype analysis indicated that the combined Foxp3 –3279 A variant genotype (CA + AA) was associated with a significantly higher Larsen score, as well as a higher means value of the VAS score without Bonferroni correction. When Bonferroni correction for multiple testing was used, we found no significant association between SNP and RA phenotype in our patients. We also found no evidence for association of the *Foxp3* –924 A/G polymorphism with the severity of RA as well as no difference in Foxp3 serum levels in relation to any of the examined *Foxp3* SNP among patients with RA and controls. However, when we compared patients with RA and the control group according to –3279 C/A and –924 A/G *Foxp3* genotypes, we found that serum levels of Foxp3 were significantly higher in patients with RA with all examined genotypes compared to healthy subjects with the same genotypes; the underlying mechanism is difficult to explain, because there are no published studies about serum/plasma Foxp3 levels. The expression level of the transcriptional factors within the target cell is a reflection of the transcriptional processes, whereas in the serum it passively reflects the undergoing regulation. That is why we postulate that the Foxp3 level in serum would depend much more on the number and activity of Treg cells, which participate in the inflammatory processes, than on the sole genotype.

Even so, the analyzed polymorphisms might still affect *Foxp3* mRNA and protein intracellular expression. Our results showing the association of these SNP with susceptibility to RA and the –3279 C/A variant with disease activity indirectly suggest disruption of Treg function as well as immune homeostasis, leading in consequence to RA. Further studies on larger patient cohorts would be desirable to reach sufficient statistical power for these observations.

Recent years have seen increasing efforts to understand the involvement of T cells in the immunopathogenesis of RA. This has led to a rising interest in the possibility of using Treg cells in biological therapy for controlling autoimmunity. Therefore, finding the genetic basis of autoimmune disorders may constitute a step toward understanding the immunology of the pathogenic process leading to the disease. It may also enable prevention of a particular disorder. Thus, the aim of our study was to fill, at least in

part, gaps in the body of knowledge, by attempting to explain some mechanisms contributing to the pathogenesis of RA and to suggest new directions in therapeutic research.

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REFERENCES

1. Lin SC, Chen KH, Lin CH, Kuo CC, Ling QD, Chan CH. The quantitative analysis of peripheral blood FOXP3-expressing T cells in systemic lupus erythematosus and rheumatoid arthritis patients. Eur J Clin Invest 2007;37:987-96.
2. Akil M, Amos RS. ABC of rheumatology: rheumatoid arthritis –I: clinical features and diagnosis. BMJ 1995;310:587-90.
3. Sangha O. Epidemiology of rheumatic diseases. Rheumatology 2000;39 Suppl 2:3-12.
4. Silman A, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis Res 2002;4 Suppl 3:S265.
5. Beavis PA, Gregory B, Green P, Cribbs AP, Kennedy A, Amjadi P, et al. Resistance to regulatory T cell-mediated suppression in rheumatoid arthritis can be bypassed by ectopic foxp3 expression in pathogenic synovial T cells. Proc Natl Acad Sci U S A 2011;108:16717-22.
6. Andersson AK, Li C, Brennan FM. Recent developments in the immunobiology of rheumatoid arthritis. Arthritis Res Ther 2008;10:204.
7. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25 (+) CD4 (+) regulatory cells that control intestinal inflammation. J Exp Med 2000;192:295-302.
8. Oda JM, Hirata BK, Guembarovski RL, Watanabe MA. Genetic polymorphism in FOXp3 gene: imbalance in regulatory T-cell role and development of human diseases. J Genet 2013;92:163-71.
9. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996;184:387-96.
10. Roncarlo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self-antigens and alloantigens in humans. Immunology 2007;7:585-98.
11. Leipe J, Skapenko A, Lipsky PE, Schulze-Koops H. Regulatory T cells in rheumatoid arthritis. Arthritis Res Ther 2005;7:93-9.
12. Yang G, Zhou H, Hickford JG. Polymorphism of the ovine FOXP2 gene (FOXP3). Vet Immunol Immunopathol 2011;140:303-6.
13. Tavakoli NN, Hambly BD, Sullivan DR, Bao S. Forkhead box protein 3: essential immune regulatory role. Int J Biochem Cell Biol 2008;40:2369-73.
14. de Paz B, Prado C, Alperi-Lopez M, Ballina-Garcia FJ, Rodriguez-Carrio J, Lopez P, et al. Effects of glucocorticoid treatment on CD25-FOXP3+ population and cytokine-producing cells in rheumatoid arthritis. Rheumatology 2012;51:1198-207.
15. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000;106:R75-81.
16. Li B, Samanta A, Song X, Furuuchi K, Iacono KT, Kennedy S, et al. FOXP3 ensembles in T-cell regulation. Immunol Rev 2006;212:99-113.
17. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. Immunology 2007;7:305-10.
18. Ziegler SF, Buckner JH. FOXP3 and the regulation of Treg/Th17 differentiation. Microbes Infect 2009;11:594-8.
19. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. FASEB J 2012;26:2253-76.
20. Peng SL. Forkhead transcription factors in chronic inflammation. Int J Biochem Cell Biol 2010;42:482-5.
21. Mays LE, Chen YH. Maintaining immunological tolerance with Foxp3. Cell Res 2007;17:904-18.
22. Sánchez E, Rueda B, Orozco G, Oliver J, Vilchez JR, Paco L, et al. Analysis of a GT microsatellite in the promoter of the foxp3/scurfin gene in autoimmune diseases. Hum Immunol 2005;66:869-73.
23. Bassuny WM, Ihara K, Sasaki Y, Kuromaru R, Kohno H, Matsuura N, et al. A functional polymorphism in the promoter/enhancer region of the FOXP3/Scurfin gene associated with type 1 diabetes. Immunogenetics 2003;55:149-56.
24. D'Amico F, Skarmoutsou E, Marchini M, Malaponte G, Caronni M, Scorza R, et al. Genetic polymorphisms of FOXP3 in Italian patients with systemic sclerosis. Immunol Lett 2013;152:109-13.
25. Wu Z, You Z, Zhang C, Li Z, Su X, Zhang X, et al. Association between functional polymorphisms of Foxp3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population. Clin Dev Immunol 2012;2012:896458.
26. Gao L, Li K, Li F, Li H, Liu L, Wang L, et al. Polymorphisms in the FOXP3 gene in Han Chinese psoriasis patients. J Dermatol Sci 2010;57:51-6.
27. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 2005;15:97-8.
28. Li Z, Zhang Z, He Z, Tang W, Li T, Zeng Z, et al. A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn). Cell Res 2009;19:519-23.
29. Bali D, Gourley S, Kostyu DD, Goel N, Bruce I, Bell A, et al. Genetic analysis of multiplex rheumatoid arthritis families. Genes Immun 1998;1:28-36.
30. Turesson C, Schaid DJ, Weyand CM, Jacobsson L, Goronzy JJ, Petersson IF, et al. The impact of HLA-DRB1 genes on extra-articular disease manifestations in rheumatoid arthritis. Arthritis Res Ther 2005;7:R1386-93.
31. Weyand CM, Goronzy JJ. Association of MHC and rheumatoid arthritis HLA polymorphisms in phenotypic variants of rheumatoid arthritis. Arthritis Res 2000;2:212-6.
32. Cucca F, Goy JV, Kawaguchi Y, Esposito L, Merriman ME, Wilson AJ, et al. A male-female bias in type 1 diabetes and linkage to chromosome Xp in MHC HLA-DR3-positive patients. Nat Genet 1998;19:301-2.
33. Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C, et al. A full genome search in multiple sclerosis. Nat Genet 1996;13:472-6.
34. Imrie H, Vaidya B, Perros P, Kelly WF, Toft AD, Young ET, et al. Evidence for a Graves' disease susceptibility locus at chromosome Xp11 in a United Kingdom population. J Clin Endocrinol Metab 2001;86:626-30.
35. Taylor JC, Gough SC, Hunt PJ, Brix TH, Chatterjee K, Connell JM, et al. A genome-wide screen in 1119 relative pairs with autoimmune thyroid disease. J Clin Endocrinol Metab 2006; 91:646-53.
36. Cornélis F, Fauré S, Martinez M, Prud'homme JF, Fritz P, Dib C, et al. New susceptibility locus for rheumatoid arthritis suggested by a genome-wide linkage study. Proc Natl Acad Sci U S A 1998;95:10746-50.
37. Han GM, O'Neil-Andersen NJ, Zurier RB, Lawrence DA. CD4+CD25high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. Cell Immunol

2008;253:92-101.
38. Lin YC, Lee JH, Wu AS, Tsai CY, Yu HH, Wang LC, et al. Association of single-nucleotide polymorphisms in FOXP3 gene with systemic lupus erythematosus susceptibility: a case-control study. Lupus 2011;20:137-43.
39. Tao YP, Wang WL, Li SY, Zhang J, Shi QZ, Zhao F, et al. Associations between polymorphisms in IL-12A, IL-12B, IL-12Rβ1, IL-27 gene and serum levels of IL-12p40, IL-27p28 with esophageal cancer. J Cancer Res Clin Oncol 2012; 138:1891-900.
40. Ryder LR, Woetmann A, Madsen HO, Ødum N, Ryder LP, Bliddal H, et al. Expression of full-length and splice forms of FoxP3 in rheumatoid arthritis. Scand J Rheumatol 2010;39:279-86.
41. Stelmaszczyk-Emmel A, Jackowska T, Rutkowska-Sak L, Marusak-Banacka M, Wąsik M. Identification, frequency, activation and function of CD4+ CD25(high)FoxP3+ regulatory T cells in children with juvenile idiopathic arthritis. Rheumatol Int 2012;32:1147-54.
42. Boissier MC, Assier E, Biton J, Denys A, Falgarone G, Bessis N. Regulatory T cells (Treg) in rheumatoid arthritis. Joint Bone Spine 2009;76:10-4.
43. Sarkar S, Fox DA. Regulatory T cells in rheumatoid arthritis. Curr Rheumatol Rep 2008;10:405-12.
44. Kao JK, Hsue YT, Lin CY. Role of new population of peripheral CD11c(+)CD8(+) T cells and CD4(+)CD25(+) regulatory T cells during acute and remission stages in rheumatoid arthritis patients. J Microbiol Immunol Infect 2007;40:419-27.
45. Fodor E, Garaczi E, Polyánka H, Koreck A, Kemény L, Széll M. The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population. Hum Immunol 2011;72:926-9.
46. Inoue N, Watanabe M, Morita M, Tomizawa R, Akamizu T, Tatsumi K, et al. Association of functional polymorphisms related to the transcriptional level of FOXP3 with prognosis of autoimmune thyroid diseases. Clin Exp Immunol 2010;162:402-6.
47. Andre GM, Barbosa CP, Teles JS, Vilarino FL, Christofolini DM, Bianco B. Analysis of FOXP3 polymorphisms in infertile women with and without endometriosis. Fertil Steril 2011;95:2223-7.
48. Park O, Grishina I, Leung PS, Gershwin ME, Prindiville T. Analysis of the Foxp3/scurfin gene in Crohn's disease. Ann N Y Acad Sci 2005;1051:218-28.
49. Raskin L, Rennert G, Gruber SB. FOXP3 germline polymorphisms are not associated with risk of breast cancer. Cancer Genet Cytogenet 2009;190:40-2.
50. Song P, Wang XW, Li HX, Li K, Liu L, Wei C, et al. Association between FOXP3 polymorphisms and vitiligo in a Han Chinese population. Br J Dermatol 2013;169:571-8.