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Research Article

IL6-174G/C (rs1800795) Polymorphism Rather than IL6R (rs2228145 and rs4845618) Polymorphisms is Associated with Susceptibility to Rheumatoid Arthritis in the Belarusian Population

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Abstract

Rheumatoid Arthritis (RA) is a chronic systemic disorder of the connective tissue of still unknown aetiology and complex autoimmune pathogenesis that primarily affects small joints.

Interleukin-6 (IL6) is a pleiotropic cytokine with a crucial role in pathogenesis of Rheumatoid Arthritis (RA). Although IL6 involvement in development and clinical outcome of rheumatoid arthritis is well established, current investigations on association between Single Nucleotide Polymorphisms (SNPs) in the IL6 gene promoter region, such as IL6 −174G/C (rs1800795) and susceptibility to RA give somewhat conflicting results.

Our preliminary study revealed significant association of IL6 −174G/C (rs1800795) with susceptibility to Juvenile Idiopatic Arthritis (JIA) in children and a higher frequency of the -174C allele in adult patients with RA, reaching statistical significance in case of RF-negative RA subtype. The aim of the present work was to evaluate association between IL6 −174G/C (rs1800795), IL6R (rs2228145, rs4845618) SNPs and RA in the Belarusian population, and determine possible correlations of these genetic variations with biological sex and autoantibody status.

In the cohort of 187 RA patients and 380 healthy donors IL6 rs1800795 CC genotype had significantly higher frequency (p = 0.0456 OR 1.52 [1.02 - 2.27], recessive genetic model) among patients with rheumatoid arthritis as compared to that in controls. No associations of IL6R rs2228145 and rs4845618 SNPs with RA susceptibility were found in the total group of patients vs. controls, however the observed frequency of homozygous genotype TT for IL6R rs4845618) was significantly lower among male patients as compared to corresponding controls and female patients. It was also shown that IL6 rs1800795 CC genotype frequency was significantly higher among the patients with RF-negative status. Thus, we provide evidence for association of the non-synonymous variant IL6 −174G/C (rs1800795) with risk of RA in the Belarusian population, some features of interplay being revealed between gene polymorphisms analyzed and both gender of patients and RA antibody status.

Keywords: Autoantibody status; Gene polymorphism; IL6; Interleukin-6; Interleukin-6 receptor; IL6R; Rheumatoid arthritis; RF-negative RA; rs1800795; rs2228145; rs4845618

Introduction

According to the data of Johns Hopkins Arthritis Center, rheumatoid arthritis has a worldwide distribution with an estimated prevalence of 1 to 2% [1]. Prevalence increases with age, approaching 5% in women over age 55. The average annual incidence in the United States is about 70 per 100,000 annually. Women are two to three times more susceptible to RA than men. Although rheumatoid arthritis may occur at any age, patients most commonly are first affected in the third to sixth decades.

In adults, arthritis is the leading cause of disability and work, physical and social activity limitations due to progressive joint damage. The number of people affected is predicted to increase by 40% in the USA over the next 25 years [2,3]. So distressing statistics and inefficient therapeutic treatment prompt scientists to search novel pathogenic pathways including molecular mechanisms underlying development of this multifactor and polygenic disease [4,5]. A significant part of the genetic component is represented by allelic variants of the highly polymorphic HLA (Human Leukocyte Antigen) locus, which (by some estimates) are found in 70% of RA patients [6]. Non-HLA genes were also shown to contribute to predisposition to this autoimmune disorder, among them, genes controlling inflammatory and autoimmune response are identified [7-9]. Due to Genome-Wide Association Study (GWAS) more than 200 genetic variants (single nucleotide polymorphisms, SNPs) were discovered to be involved in susceptibility to RA, and their range is permanently broadened [9]. It is typical, that most of genes are common for Caucasian and Asian populations, but some of them are specific to the Europeans, while

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others are specific to Asians. The cumulative data have indicated urgent need to study the problem of susceptibility to RA in the concrete ethno-geographic conditions.

As an inflammatory disease, RA is characterized by increased levels in pro-inflammatory cytokines, which are secreted by immune cells and are vital part of the immune network to provide communication [10-12]. Interleukin-6 (IL6), a pleiotropic cytokine with multiple functions in different pathophysiologic systems including rheumatoid arthritis (RA), has a high priority among pro-inflammatory cytokines, fulfilling such functions as osteoclast and B-cell activation, T-cell proliferation and differentiation, acute-phase protein production [13]. High concentrations of IL6 in both the synovial fluid and serum of patients with RA suggested a major role of this cytokine in RA pathogenesis [14].

As a rule, cytokines carry out the functions interacting with specific receptors. Their level in biological fluids is known to be determined by expression of corresponded genes, which may be modulated by SNPs in the promoter region. Such effects are inherent, for example, to IL6 −174G/C polymorphism (rs1800795) [15]. However, the data regarding IL6 levels across −174G/C variants are controversial; some studies have shown higher circulating IL6 levels in GG carriers, whereas other studies have reported no difference between genotypes or even increased levels in CC carriers, suggesting the complicated mechanism in determining circulating IL6 level [15].

Impact of IL6 and IL6R polymorphisms on probability of developing RA was also studied with varying success. Although some case-control studies indicated increasing RA risk in carriers of the −174G/C (rs1800795) IL6 polymorphism, others, including the meta-analysis of GWAS data by Okada Y et al., have shown that no IL6 locus SNPs are associated with RA [8,16]. Our preliminary investigation revealed statistically significant association of the −174G/C IL6 polymorphism with juvenile idiopathic arthritis in children and a trend to higher frequency of the minor C allele in adult patients with RA [17].

In the light of all above, it was interesting to study the role of IL6 and IL6R polymorphisms (rs1800795; rs2228145; rs4845618) in developing RA in Belarusian population taking into account possible modulation of genetic effects due to etiologically important factors such as gender (biological sex) of patients and the antibody status.

Materials and Methods

Study groups

One hundred and eighty-seven Belarusian patients with RA (cases) and 380 healthy individuals without chronic inflammatory and autoimmune diseases (controls: F/M: 285/95, mean age 37.18 ± 10.69 years) were recruited in the Scientific-Practical Center of Surgery, Transplantation and Hematology (Minsk). Patients with RA were included into the case-group due to the verified diagnosis of RA according classification diagnostic criteria of the American College of Rheumatology (1987, ACR; formerly, the American Rheumatism Association) and the classification criteria by ACR\EULAR 2010 [18,19]. Detailed diagnosis was made on the basis of clinical and laboratory instrumental examinations. Informed consent was obtained from all participants, who were also interviewed for some demographic characteristics, pernicious habits and anamnesis. Clinical and serological (RF and anti-CCP antibody status) data on RA patients were collected from individual disease history. RA was deemed seropositive in case of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies' serum levels exceeded reference values (for RF positivity:≥30 IU/ml, for anti-CCP positivity: ≥5 U/ml).

SNP genotyping

Total genomic DNA was isolated from peripheral blood samples using standard phenol-chloroform DNA extraction protocol [20]. Samples were genotyped for selected SNPs by real-time PCR using fluorescent probes (Primetech ALC, Minsk, Belarus) (Table 1). Rs1800795 (which is more often referred to as "−174G/C") is located in the promoter region (−174) of IL6 gene. Among two selected SNPs of IL6R, rs2228145 is a missense variant p.Asp358Ala and rs4845618 is an intronic variant. All PCR and endpoint fluorescent readings were performed using Bio-rad CFX 96 real-time PCR detection system (Bio-Rad, Hercules, CA). Thermal cycling was initiated at 95°C for 10 min, followed by varying number of cycles of denaturation at 95°C for 15 sec, annealing at SNP-specific t°C for 30 sec and extension at 72°C for 30 sec.

Statistical analysis

Allele and genotype frequencies of IL6/IL6R SNPs were obtained by direct counting. For allele and genotype frequency comparisons, we used $\chi 2$ test with 2×2 contingency tables. Odds Ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using Vassar Stats online tool [21]. Genetic model analysis was further utilized to confirm the association of the genotype. P-values of < 0.05 (two-tailed) were considered statistically significant.

Results

A total of 187 samples from RA patients were analyzed for association between IL6 and IL6R SNPs (rs1800795, rs4845618 and rs2228145) and risk of developing RA. IL6 rs1800795 was successfully genotyped in 380 healthy controls, whereas rs4845618 and rs2228145 were genotyped in 324 and 284 controls respectively. Demographic and serological characteristics of patients diagnosed with RA are summarized in table 2.

SNP	Forward primer, 5′→3′	Reverse primer, 5′→3′	Probe, 5′→3′	Annealing, (°C)
IL6 rs1800795	CGACCTAAGCTGCACTTTTCC	GGGCTGATTGGAAACCTTATTAAGATTG	FAM– CCTTTAGCATCGCAAGAC	62
			ROX –CCTTTAGCATGGCAAGAC	
IL6R rs2228145	TCTCCTCTTCCTCCTCTATCT	CAGGCTCCCTCCAGCAA	FAM– AGCTTCTTCTTCAGTACCACTG	61
			ROX –AGATTCTTCTTCAGTACCACTG	
IL6R , rs4845618	CTGCACGGTGACAATTCAAC	CTTCACACTTCCCTCTCACTTTA	FAM –CTTATATCTGTCCCTTTTCGCC	55
			ROX – CTTATATCTTTCCCTTTTCGCC	

Table 1: Summary of oligonucleotide primers/probes and annealing temperature used for real-time PCR-based genotyping.

Characteristics	Value
No. of patients	187
Age, mean ± SD years	58.2 ± 11.9
Disease duration, mean ± SD years	13.2 ± 11.1
Female no. (%)	148 (79)
Male, no. (%)	39 (21)
Rheumatoid factor positive, RF (+), no. (%)	117 (62.6)
Rheumatoid factor negative, RF (-), no. (%)	50 (26.7)
No RF data available, no. (%)	20 (10.69)
Anti–cyclic citrullinated peptide antibody positive, anti-CCP (+), no. (%)	144 (77.0)
Anti–cyclic citrullinated peptide negative, anti-CCP (-), no. (%)	35 (18.7)
No ACCP data available, no. (%)	8 (4.28)

Table 2: Demographic characteristics and serological status of RA patients included in the study.

The data show that the total sample of patients is predominantly represented by women (79%), the average age is 58.2 ± 11.9 years. RF and anti-CCP levels were evaluated in 167 and 179 patients, respectively. RF and anti-CCP antibody positivity usually coincide, but there were 18 cases when anti-CCP (+) combined with RF (-), and combination of RF (+) with anti-CCP (-) was observed in 5 cases.

Genotype/allele frequency distributions were as shown in table 3. Utilizing recessive genetic model we found an association between CC genotype of IL6 rs1800795 and RA as compared to controls (OR = 1.52 [1.02 - 2.27] p 0.0456; table 3). Stratification for gender of the patients with RA had shown that the potential RA risk CC genotype of rs1800795 also manifested in female patients when compared with female controls.

When genotyping IL6R rs4845618 and rs2228145 loci, no significant differences were revealed in the distribution of the allele/genotype frequencies. Results of analysis for IL6R SNPs in the RA group were consistent with data on allele/genotype frequency distribution of these variants in Belarusian children with JIA [17]. However, there were differences in distribution of TT genotype frequencies of IL6R rs4845618 in men: TT genotype frequencies were somewhat lower in male patients versus corresponding controls and when comparing with female patients. Similar data were observed in relation to frequency distribution of IL6R rs2228145 CC genotype (Table 3).

At the next step of our investigation we attempted to analyze a possible association of studied SNPs with RA clinical parameters, namely serological status (Table 4). The data indicated that CC genotype of IL6 rs1800795 was associated with RF (-) RA. The rest of genetic variants were neutral with respect to RA risk independently of serological status of patients.

Discussion

Our case-control study demonstrated an association of IL6 promoter -174G/C (rs1800795) polymorphism with increased RA risk. The carries of the CC genotype were more susceptible to developing RA (OR = 1.52 [1.02 - 2.27] p 0.0456), and this difference was also significant in women. In the same time, studied SNPs in IL6R locus showed no association with RA in the total cohort with inverse effects in men (p = 0.074 OR=0.15 [0.0193 - 1.2007] for the CC genotype of rs2228145 in men as compared to corresponding controls and p = 0.039 OR=0.30 (0.0981 - 0.9375) for TT genotype of rs4845618.

As mentioned above, the data concerning IL6 SNPs associations with RA are contradictory. There are similar conflicting data with respect to IL6 levels affected by IL6 −174G/C (rs1800795). Marinou et al. found that the IL-6 -174 major allele is associated with radiographic damage and disease activity [22]. However, RF and anti-CCP status was not associated with IL6 genotypes.

SNP	Geno-type/ allele	Total		Men		Women	
		RA patients, n (%)	Controls, n (%)	RA patients, n (%)	Controls, n (%)	RA patients, n (%)	Controls, n (%)
rs1800795 (IL6)	GG	50 (26.46)	112 (29.47)	11 (28.2)	30 (31.58)	39 (26)	82 (28.77)
	GC	85 (44.97)	189 (49.74)	17 (43.6)	42 (44.21)	68 (45.33)	147 (51.58)
	CC	**54 (28.57)[1]**	79 (20.79)	11 (28.2)	23 (23.21)	**43 (28.67)[2]**	56 (19.65)
	G	185 (48.94)	413 (54.34)	39 (50)	102 (53.68)	146 (48.67)	311 (54.56)
	C	193 (51.06)	347 (45.66)	39 (50)	88 (46.32)	154 (51.33)	259 (45.44)
rs2228145 (IL6R)	AA	80 (42.33)	118 (41.5)	19 (48.72)	36 (37.89)	61 (40.67)	82 (43.38)
	AC	91 (48.15)	128 (45.1)	19 (48.72)	45 (47.37)	72 (48)	83 (43.92)
	CC	18 (9.52)	38 (13.4)	**1 (2.56)[3]**	14 (14.74)	17 (11.33)	24 (12.7)
	A	251 (66.4)	364 (64.1)	57 (73.08)	117 (61.58)	194 (64.67)	247 (65.34)
	C	127 (33.6)	204 (35.9)	21 (26.92)	73 (38.42)	106 (35.33)	131 (32.66)
rs4845618 (IL6R)	GG	43 (22.75)	78 (24.1)	11 (28.2)	22 (23.16)	32 (21.33)	56 (24.45)
	GT	102 (53.97)	167 (51.5)	24 (61.54)	47 (49.47)	78 (52)	120 (52.4)
	TT	44 (23.28)	79 (24.4)	**4 (10.26)[4,5]**	26 (27.37)	40 (26.67)	53 (23.15)
	G	188 (49.74)	323 (49.85)	46 (58.97)	91 (47.89)	142 (47.33)	232 (50.65)
	T	190 (50.26)	325 (50.15)	32 (41.03)	99 (52.11)	158 (52.67)	226 (49.35)

Table 3: Case-control analysis of distribution of genotype\allele frequencies of studied SNPs also stratified by sex.

[1] - recessive genetic model (rec.), overall cases vs. controls p = 0.0456 OR=1.52 95%CI [1.02 - 2.27]; [2] - rec., female patients vs. female controls p = 0.04 OR=1.64 95%CI [1.039 – 2.60]; [3] - rec., male patients vs. male controls p = 0.074 OR=0.15 95%CI [0.0193 – 1.2007]; [4] - rec., male cases vs. male controls p = 0.039 OR=0.30 95%CI [0.0981 – 0.9375]; [5] – rec., male patients vs. female patients p = 0.033 OR=0.31 95%CI [0.105 - 0.9404]

	Genotype/ allele	RF+, n (%)	RF-, n (%)	anti-CCP+, n (%)	anti-CCP-, n (%)	Controls, n (%)
rs1800795 (IL6)	GG	34 (29.06)	11 (22)	34 (23.61)	12 (34.29)	112 (29.47)
	GC	54 (46.15)	20 (40)	68 (47.22)	15 (42.86)	189 (49.74)
	CC	29 (24.79)	**19 (38)[1]**	42 (29.17)	8 (22.85)	79 (20.79)
	CC+GC	83 (70.94)	39 (78)	110 (76.39)	23 (65.71)	268 (70.53)
	C	112 (47.86)	58 (58)	152 (52.78)	31 (44.29)	347 (45.66)
rs2228145 (IL6R)	AA	50 (42.74)	23 (46)	63 (43.75)	15 (42.86)	118 (41.5)
	AC	58 (49.57)	19 (38)	66 (45.83)	17 (48.57)	128 (45.1)
	CC	9 (7.69)	8 (16)	15 (10.42)	3 (8.57)	38 (13.4)
	CC+AC	67 (57.26)	27 (54)	81 (56.25)	20 (57.14)	166 (58.5)
	C	76 (32.48)	35 (35)	96 (33.33)	23 (32.86)	204 (35.9)
rs4845618 (IL6R)	GG	24 (20.51)	14 (28)	31 (21.53)	10 (28.57)	78 (24.1)
	GT	67 (57.26)	21 (42)	80 (55.56)	15 (42.86)	167 (51.5)
	TT	26 (22.22)	15 (30)	33 (22.92)	10 (28.57)	79 (24.4)
	TT+GT	93 (79.49)	36 (72)	113 (78.47)	25 (71.43)	246 (75.93)
	T	119 (50.85)	51 (51)	146 (50.69)	35 (50)	325 (50.15)

Table 4: Distribution of IL6, IL6R genotypes/alleles among Belarusian RA patients with varying serological status as compared to healthy controls.

RF+ - Rheumatoid Factor positive; RF- - Rheumatoid Factor-negative; anti-CCP+ - anti-CCP Antibody-Positive; anti-CCP- - anti-CCP Antibody-Negative.

[1] - Genotypic test, CC IL6 rs1800795 in RF (-) RA patients vs. controls p = 0.0019

In Polish patients, the frequency distribution in the same locus (rs1800795) was similar in the RA and control groups, but homozygotes by the minor C allele associated with highest average concentration of the cytokine and disease activity. On this ground, the authors have concluded that CC homozygosity may play a rather unfavorable role in RA [23].

Although a majority of independent investigations in different European populations did not reveal the positive association of the IL6 rs1800795 polymorphism with RA, meta-analysis of Asian populations demonstrated such association (OR = 9.75, 95%CI [4.99-19.06], p <0.00001) [24]. The data on European and Asian populations were included in further meta-analysis which showed the statistically significant association between IL6 (rs1800795) -174 ***C*** allele and RA in the combined population (OR = 1.59; 95%CI: 1.19-2.14, P < 0.05), the similar effect for GC+ CC genotypes [15]. In Asians, positive associations were found in carriers of −174C allele as well as for CC and GC+CC genotypes. Therefore, the contribution of the IL6 rs1800795 minor allele to developing RA seems quite convincing.

A set of individual case-control studied indicated the positive association between the level of circulating IL6 cytokine and its soluble receptor and RA [25-29]. However, the data on how the IL6 rs1800795 variant affects the level and activity of the cytokine are contradictory. For example, JIA patients, carriers of the major G allele had the increased level of this cytokine [30]. On the other hand, levels of IL6 cytokine were increased in RA adult patients, carriers of the C minor allele [31]. Utilizing Mendelian randomization, Bing Li et al. concluded that "carriers of the IL6 -174 GC+CC genotype showed significant reduction in the circulating IL-6 level (WMD = -0.77; 95% CI: -1.16 to -0.38; p = 0.000) when compared with carriers of GG genotype. Conversely, a significantly increased circulating IL6 level (WMD = 0.64; 95% CI: 0.26-1.03; p = 0.001) was observed in carriers of GG+GC genotype compared with carriers of the CC genotype" [15]. Seemingly in contrast with these effects, the IL6 -174 minor allele and relative genotypes were risk variants for developing RA [15]. Thus, a negative causal relevance of circulating IL6 level with RA risk in overall and Asian populations was demonstrated that indicated intricate regulation of IL6 gene expression contributing to disease development.

Investigations by Noss et al., and Isomaki et al., provided some information elucidating this genetic puzzle [32,33]. Noss et al., reported a striking reproducible association between the IL6 promoter SNP rs1800795 CC genotype and high IL6 production by synovial fibroblasts and absence of such association in monocytes. The data obtained indicated the alternative effects of IL6 polymorphisms depending on cell types and a prominent impact of IL6 SNP rs1800795 on regulation of this cytokine in fibroblasts [32]. Isomaki et al., demonstrated a higher level of circulating IL6 in blood of patients with RA as compared with healthy volunteers due to constitutive activation of transcription STAT3 [33]. However, IL6-induced STAT3 phosphorylation was found to occur only in a certain fraction of T cells from RA patients whereas in some patients the response to IL6 stimulation was comparable to that in healthy controls. By authors' opinion, these results show that the IL6/STAT3 pathway is involved in the initiation and progression of RA, but in individual-dependent manner.

Additionally, although RF status and anti-ACPA status were not associated with genetic variants of IL6 (rs1800795), we revealed a positive association between the IL6 proximal promoter SNP rs1800795 CC genotype and RF-negative status [22]. In support of our data, some studies has shown that IL6-driven STAT signaling is observed particularly in early ACPA negative patients [34,35]. In this context, it should be noted that significant association was observed between IL6 -174C allele and early disease onset of RA [36].

In conclusion, when taking together the literature findings and our own data, it seems that importance of IL6 (rs1800795) −174C allele and its related genotypes in development of RA is rather convincing. However, further investigations to elucidate complex mechanisms of interleukin-6/interleukin-6 receptor networking in development of RA are required.

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Conflict of Interest

The authors ES Siniauskaya, TD Kuzhir, VE Yagur, RI Goncharova declare that they have no conflict of interest to disclose.

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