

Original article

Relating interferon regulatory factor 5 rs2004640 gene polymorphism to increased risk of systemic sclerosis in the patients: Russian Federation cohort

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Abstract: *Background* — A number of studies confirmed a crucial role of type 1 interferon in pathophysiology of connective tissue diseases. Interferon regulatory factors (IRF) coordinate an expression of type 1 interferon, while interferon regulatory factor 5 (IRF5) gene was recently identified as causing predisposition to systemic lupus erythematosus and Sjögren syndrome.

The objective of our study was to identify possible association of IRF5 rs2004640 (G/T) single nucleotide polymorphism with systemic sclerosis (SSc).

Material and Methods—The study involved 236 individuals, including 105 patients with SSc diagnosis and 131 control individuals from Moscow region. The latter were healthy, unrelated to each other, their genders and ages were matched to those of SSc patients. Allele-specific real-time polymerase chain reaction (PCR) was used to study IRF5 rs2004640 polymorphism.

Results — We detected significantly higher percentage of IRF5 T-allele carriers in all patients (59.5%), those with diffuse cutaneous SSc (67.3%), patients with interstitial lung lesions (62.3%), and those with positive titers of anti-topoisomerase I antibodies (66.3%), compared with control group (46.2%). The odds ratios (OR) were: 1.71 ($p=0.00$), 2.40 ($p=0.0004$), 1.93 ($p=0.002$), and 2.30 ($p=0.0008$), correspondingly.

Conclusion — The replacement of nucleotide G by T in the IRF5 rs2004640 gene polymorphism was associated with a predisposition to SSc. Our data implied an existence of a novel SSc pathogenetic pathway associated with important role of type 1 interferon in pathophysiology of connective tissue diseases.

Keywords: interferon, IRF5 gene polymorphism, systemic sclerosis, interstitial lung disease.

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Introduction

Systemic sclerosis (SSc) is a chronic multisystem connective tissue disorder characterized by immune system dysfunction associated with production of autoantibodies; early development of generalized vasculopathy; extensive deposition of collagen and other matrix proteins in connective tissues; and fibrosis of internal organs. SSc is linked to a higher mortality rate compared with other rheumatic diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or Sjögren syndrome (SS) [1]. The most common causes of mortality associated with SSc include interstitial lung disease (ILD) and pulmonary hypertension (PH). The exact etiology of SSc and accompanying ILD is currently unclear, but clinical and experimental data confirm multifactorial pathogenesis of this disease involving genetic and environmental factors [2]. Recent genetic studies confirmed that a number of candidate genes and their single nucleotide polymorphisms (SNPs) could be risk factors for SSc [3].

Type 1 interferon (IFN-1) is a central regulator of natural immunity, and its production is an ordinary physiological immune response to microbial infections. It stimulates various physiological

processes: maturation of monocytes from dendritic cells, maturation of plasma cells, switching in immunoglobulin synthesis, activity of cytotoxic T cells and natural killer cells, and secretion of chemokines [4, 5]. Studies involving microchips have shown an important role of IFN-1 in pathophysiology of SLE and SS [6, 7], as well as of other autoimmune disorders, such as dermatomyositis (DM), psoriasis (Ps), RA [8] and (as it was recently shown) SSc [9]. IRF5 is an important regulator of IFN-1 signal transmission. A large-sample French case-control study established an association between IRF5 rs2004640 gene polymorphism and SSc, especially dcSSc (diffuse cutaneous systemic sclerosis) subtype [9]. In a Japanese study [10], the authors confirmed the association of IRF5 rs2004640 polymorphism with SSc, and also discovered additional associations of rs10954213 and rs2280714 polymorphisms with SSc, which were most pronounced in the patients with dcSSc phenotype and anti-topoisomerase I autoantibodies seropositivity (ATA+). In Dieude et al. study [11] of three SNPs in IRF5 rs3757385, rs2004640 and rs10954213 genes, the authors found two haplotypes designated as “R” (C-T-A) – a “risk haplotype” associated with dcSSc subtype of systemic sclerosis, and “P” (A-G-

G) – a “protective haplotype”. The haplotypes were associated with both cutaneous SSc subtypes and fibrosing alveolitis. Several other genome studies have also confirmed the association of IRF5 with SSc [12, 13]. Up to date, the role of IRF5 rs2004640 polymorphism in the Russian Federation population has not been studied in terms of its association with clinical and autoimmune phenotypes, as well as in terms of causing the patients’ predisposition to SSc development.

An objective of our study was to investigate potential association of IRF5 rs2004640 (G/T) gene polymorphism with susceptibility of the Russian Federation population to SSc.

Material and Methods

Study design

Our case-control study involved 236 people; including 105 patients with SSc diagnosis (13 males, 12.4%, and 92 females, 87.6%) and a control group of 131 healthy, unrelated to each other individuals from Moscow region, their genders and ages matched to those of SSc patients. All SSc patients on the record at V.A. Nasonova Institute of Rheumatology clinic from September 2011 to January 2014 had an average age of 49.8±12.4 yr and average disease duration of 11.2±9.3 yr. All SSc patients meet 1980 American College of Rheumatology (ACR) criteria for the SSc classification [14]. Patients with typically manifest SSc represented the study group. All patients in the study group manifested at least three of five CREST syndromes (calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias). Based on medical documentation (diagnosis at discharge), all patients were stratified by their clinical phenotypes. None of the control group individuals had any autoimmune disorders.

Computed tomography of the lungs

Pulmonary fibrosis in the patients was established on the basis of characteristic pattern of the data obtained viaspiral computed tomography of the chest: presence of ILD was interpreted as ILD(+).

Immunological Assays

Autoimmune phenotypes included patients with positive serum titers of antinuclear autoantibodies (ANA+), anti-topoisomerase I antibodies (ATA+) or anti-centromere antibodies (ACA+). Indirect immunofluorescence on HEp-2 cells (II-HEp-2) with “IMMCO Diagnostics”, USA, reagent kit was used to detect ANA and ACA. ATA was identified by enzyme-linked immunosorbent assay (ELISA) using “Orgentec” reagent kit (Germany). ANA and ATA titers >1/160 U/ml and ATA >25.0 U/ml were considered positive outcomes.

Genotyping Assay

Samples of venous blood were obtained from all participants. DNA was isolated from fresh or frozen blood samples using “GS-genetics” kit manufactured by “DNA Technology” (Moscow, Russia). We used allele-specific real-time PCR to study IRF5 rs2004640 polymorphism. Labeled primers and probes, amplification conditions were synthesized and developed by “SYNTOL” CJSC (Moscow, Russia). We have also used DT-96 amplifier by “DNA Technology” (Moscow, Russia). Ten percent of

analyzed DNA samples from SSc patients were sequenced to confirm 100% match with genotyping outcomes.

V.A. Nasonova Institute of Rheumatology Ethics Committee approved the study, and written informed consent was obtained from all patients.

Statistical analysis

Differences in genotype frequency distribution between the patients and control individuals were assessed using 2 x 2 contingency tables and Fisher’s exact test. The most common GG genotype was used as reference. Clinical phenotypes were presented as dichotomous variables. Our data on age and ailment duration were presented in the form: mean ± standard deviation (m±SD), and percentages were analyzed. We used ANOVA *post hoc* test for analyzing the relationship among dichotomous variables and IRF5 gene polymorphism. The odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated. Frequency distributions accordance Hardy-Weinberg law in the control group were tested using the chi-square (χ^2) test with one degree of freedom. A value of $p < 0.05$ was considered statistically significant. Two-tailed p-values (Fisher’s exact test) were computed for processing small-value variables. The analysis was performed using Statistica 6.1 software package (StatSoft Inc, Tulsa, USA).

Results

Patient traits

Demographics, along with clinical and immunological phenotypes of assessed SSc patients are presented in *Table 1*.

When using the chi-square (χ^2) test, distribution of genotype frequencies in the control group was consistent with Hardy-Weinberg law.

IRF5G/T variants, and SSc clinical and serological phenotypes.

We found significant differences in the distribution of IRF5 T-allele frequencies between SSc patients and control subjects: $\chi^2=8.1$, $p=0.017$ (*Table 2*). IRF5 T-allele frequency in SSc patients was 59.5% compared to 46.2% in control subjects [OR 1.71, 95% CI (1.17-2.52), $p=0.004$].

Table 1. Demographic and clinical phenotypes of systemic sclerosis patients

Traits	SSc patients n=105	Control n=131
Gender: Male/Female	13/92	17/114
Age, years, mean (±SD)	49.8±12.4	51.3 ± 13.6
Disease duration, years, mean (±SD)	11.2± 9.3	-
≤3 years (M±δ)	11(10.8)	-
>3 years (M±δ)	91 (89.2)	-
lcSSc n (%)	53 (51)	-
dcSSc n (%)	52 (49)	-
ILD(+) n (%)	81 (77)	-
ANA(+) n (%)	88 (84)	-
ATA(+) n (%)	52 (50)	-
ACA(+) n (%)	17 (15)	-

lcSSc, limited cutaneous SSc subtype; dcSSc, diffuse cutaneous SSc subtype; ILD(+), interstitial lung disease; ANA(+), antinuclear autoantibodies positivity; ATA(+), anti-topoisomerase I antibodies positivity; ACA(+), anti-centromere antibodies positivity.

Table 2. The distribution of genotypes and IRF5 allele frequencies in all SSc patients and in subgroups by clinical and serological phenotypes vs the control group.

IRF5 G/T gene	Genotypes			Total	Frequency of T allele n (%)	OR 95%CI	p
	GG n (%)	GT n (%)	TT n (%)				
Control	39 (29.8)	63 (48.1)	29 (22.1)	131	121 (46.2)		
lcSSc	20 (19.0)	45 (42.8)	40 (38.2)	105	125 (59.5)	1.71 (1.17-2.52)	0.004
dcSSc	13 (24.5)	24 (47.3)	15 (28.3)	53	55 (51.9)	1.26 (0.78-2.03)	NS
ILD(+)	7 (13.5)	20 (38.5)	25 (48.1)	52	69 (67.6)	2.40 (1.45-3.99)	<0.001
ANA(+)	10 (12.3)	41 (50.6)	30 (37.0)	81	101 (62.5)	1.93 (1.27-2.94)	0.012
ATA(+)	12 (13.8)	38 (44.7)	37 (42.5)	87	112 (64.4)	2.11 (1.39-3.16)	<0.001
SSc	4 (7.8)	26 (51.0)	21 (41.1)	51	68 (66.7)	2.30 (1.40-3.81)	<0.001

p, patients vs control (for T-allele frequencies); statistically significant results are in bold; lcSSc, limited cutaneous SSc subtype; dcSSc, diffuse cutaneous SSc subtype; ILD(+), interstitial lung disease; ANA(+), antinuclear autoantibodies positivity; ATA(+), anti-topoisomerase I antibodies positivity; OR, odds ratio; 95% CI, 95% confidence interval; NS: not significant.

The IRF5 TT genotype was detected in 38.2% of SSc patients compared to 22.1% in the control group [OR 2.16, 95% CI (1.18-3.99), p=0.011]. In the patients with SSc duration of over 3 years, the IRF5 T-allele and IRF5 TT genotype frequencies were higher compared to the control group (63.1% and 46.2%, p=0.001; 41.3% and 20.1%, p=0.005, respectively; these results are not presented in tables). There was no correlation of this polymorphism with gender and age.

Analysis of variance revealed statistically significant differences in the frequencies of the IRF5 T allele between the groups of patients with dcSSc, ILD(+), ANA(+), ATA(+) and the control group (Table 2). IRF5 T-allele carriers in the patient group were 2.4 times more susceptible to develop dcSSc, compared to carriers of the corresponding allele in the control group [OR 2.40, 95% CI (1.45-3.99), p=0.0004]. In this SSc form, the frequency of the IRF5 TT genotype among patients was also increased, compared to control subjects [OR 3.26, 95% CI (1.55-6.80), p=0.001]. No reliable associations were found in the frequencies of the IRF5 T allele between lcSSc (limited cutaneous systemic sclerosis) patients and control subjects.

Analysis of our data showed an association between IRF5 T-allele carriers and ILD(+). IRF5 T-allele carriers were 1.9 times more likely to develop SSc-related ILD (Table 2), compared with control. Homozygous mutant genotype frequency of the patients with this clinical phenotype was also significantly increased (Table 2). No significant differences were found in distribution of IRF5 T-allele frequencies in patient groups with ILD(+) vs. ILD.

IRF5 T-allele carrier frequency in the patients with ANA(+) phenotype was also significantly different from the corresponding frequency in the control group [OR 2.11, 95% CI (1.39-3.19), p=0.0001]. Frequency of the IRF5 TT genotype among ANA(+) patients was also higher than in control (Table 2). However, we detected no differences when comparing IRF5 T-allele frequencies with homozygous genotype in ANA(-) group vs. control group. There were no significant differences in frequency distribution of IRF5 T allele vs. IRF5 TT genotype in the ANA(+) and ANA(-) patient groups.

IRF5 T-allele frequency was significantly higher in ATA(+) patients (66.3%) vs. control (p=0.0005). IRF5 T-allele carriers were 2.3 times more likely to form ATA(+) serological SSc phenotype in patients compared with control (Table 2). The frequencies of the IRF5 TT genotype also significantly differed between the patients and control subjects (40.4% vs. 22.1%, p=0.012). We found no differences when comparing the frequencies of the IRF5 T allele

between the ATA(-) group and control. There were no significant differences in frequency distributions of the T allele and TT genotype in the patient groups with ATA(+) and ATA(-).

Comparison of T-allele frequencies in ACA(+) subgroup vs. the controls was not feasible due to small ACA(+) sample size (17 patients).

Discussion

Our results reliably confirmed the association between IRF5 rs2004640 polymorphism and SSc, therefore implying that we could consider it a SSc susceptibility gene. Our results were consistent with Dieude et al. [9] data arising from a large-sample study, in which IRF5 rs2004640 functional polymorphism was genotyped in 1641 subjects of French European Caucasian origin: an original set of 427 patients with SSc and 380 control subjects, and a replication set comprising of 454 patients with SSc and 380 control subjects. The authors showed that, in both samples, the IRF5 TT genotype was significantly more common in patients with SSc than in control subjects. For combined cohort, OR=1.58, 95% CI=(1.18-2.11), p=0.002. Analysis of SSc sample in France showed a significant association between homozygosity for the IRF5 T allele and the presence of dcSSc, fibrosing alveolitis, ANA(+), and ATA positivity phenotype. The similarity in distribution of IRF5 rs2004640 alleles' and genotypes' polymorphism frequencies in the control groups of both studies (French and Russian) is worth mentioning. Current study demonstrated significant associations of IRF5 T allele with various phenotypes: dcSSc subtype (p=0.0004); ILD(+), p=0.002; ANA(+), p=0.0003; and ATA(+), p=0.0008.

An important role of three IRF5 SNP-haplotypes (rs10488631, rs2004640, and rs4728142) as susceptibility factors to SSc was reported in a multicenter study by Carmona et al. [15] with no specific associations with SSc phenotypes. Higher frequency of homozygous IRF5 TT genotype associated with lcSSc, pulmonary fibrosis, ATA(+) and ACA(+) was established for Chinese patients [16]. In the meta-analysis, Xu et al [17] showed the association of IRF5 rs2004640 polymorphism and rs7574865 polymorphism in signal transducer and activator of transcription (STAT4) genes with susceptibility to SSc, lcSSc and dcSSc variants. Another meta-analysis of eight SSc-associated SNP polymorphisms showed that solely IRF5 rs2004640, STAT4 rs7574865, IRAK1 rs1059702 and CTGF rs945 gene polymorphisms were associated with SSc and pulmonary fibrosis [18]. Since the first three polymorphisms were important regulatory control factors of innate immunity, and

theCTGF gene, encoding the connective tissue growth factor, was involved in regulation of extracellular matrix synthesis, the results obtained by those authors confirmed the role of innate immunity and matrix components in pathogenesis of SSc-associated pulmonary fibrosis.

Lungs are among the targets of fibrosis process under SSc. We noticed a strong association between ILD(+) cases and IRF5 T allele, which was characteristic for 62.3% of the patients [OR 1.93, p=0.002] and 37% of IRF5 TT homozygous genotype carriers [OR 2.07, p=0.028]. In other words, it can be assumed that IRF5 rs2004640 gene polymorphism is a susceptibility factor for ILD development, although it is not a factor determining the severity of the ailment. A number of recent studies have shown that lung involvement in SSc is a multifactorial process, including sociodemographic and behavioral factors in addition to genetic causes [19].

The regulatory factor IRF5 coordinates the expression of IFN-1, which plays an important role in the pathophysiology of connective tissue diseases, such as SS, SLE and SSc [20]. Allele T of rs2004640 polymorphism creates a donor splicing site in one intron of the gene sequence transforming it in alternative 1B exon.

Available literature presents evidence of occurrence of SSc-like phenotype and adverse events in the patients receiving interferon- α or interferon- β therapy in randomized and placebo-controlled trials [21]. It was noted that patients receiving active therapy in the form of subcutaneous injections of interferon- α , had more severe lung function disorders and a tendency to skin progression, compared with the patients receiving placebo. Published data show the involvement of interferon- α and the IRF5 gene in pathophysiology of SSc and especially in ILD phenotype. Participation of the IRF5 gene as a genetic factor in predisposition to various autoimmune ailments seems to be important for understanding its role in interferon-dependent autoimmunity.

Previously, in a similar cohort of SSc patients, when studying the effect of rs7574865 (G/T) polymorphism of the STAT4 gene on the risk of developing SSc, we obtained similar results [22]. The STAT4 gene encodes the STAT-4 protein, which transmits signals from interleukins-12, 23, and interferon-1 into T-cells and monocytes. A significant association was shown for the rs7574865 T-allele polymorphism of the STAT4 gene with a predisposition to SSc and a number of its clinical variants: dsSSc, ILD(+), ATA (+). Thus, replacing G/T in rs2004640 and rs7574865 polymorphisms of the IRF5 and STAT4 genes lead to predisposition of mutant allele carriers of these genes to SSc. These data established that genetic polymorphisms can play a role in development of SSc and provide a new understanding of SSc pathogenesis.

Conclusion

Thus, the presented data exhibited reliable association between IRF5 rs2004640 gene polymorphism and SSc. We established that T allele was a risk factor causing the predisposition to dcSSc, ILD, and seropositivity to anti-topoisomerase I antibodies. Our data implied a novel pathway for genetic factors participation in forming susceptibility to SSc and confirmed the IRF5 gene role in the genetic map of this disease.

Limitations

The data presented in a current study have a number of limitations related to the small sample size of the patients with

SSc, simultaneous case-control investigation, and require confirmation using large sample sizes of patients from different ethnic cohorts.

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Authors' contributions

M.K. conducted genotyping assay; L.A. and I.G. recruited the patients and were involved in clinical studies; I.G. performed statistical analysis; M.K. and L.A. developed the study concept and design, coordinated the research and participated in draft manuscript preparation. All authors approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical approval

All procedures performed in our studies involving human participants were in accordance with the standards of the Ethics Committee at V.A. Nasonova Research Institute of Rheumatology (Moscow, Russia), as well as with 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

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