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The *IRF5* rs2004640 (G/T) polymorphism is not a genetic risk factor for systemic lupus erythematosus in population from south India

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***Background & objectives*: Genetic aberrations disrupting toll-like receptor and interferon homeostasis enhance the risk of systemic lupus erythematosus (SLE). Raised serum interferon-alpha (IFN-α) levels in SLE patients have been ascribed to polymorphism (rs2004640 G/T) in interferon regulatory factor 5 (*IRF5*) gene, resulting in enhanced transcript splicing. A positive association between *IRF5* polymorphism and SLE risk has been reported in many populations. This study was aimed to find out frequency of *IRF5* rs2004640 G/T polymorphism in patients with SLE and healthy controls and to assess its influence on susceptibility, clinical and serological characteristics of SLE.**

***Methods*: *IRF5* rs2004640 (G/T) polymorphism was analyzed in 300 SLE patients and 460 age and sex matched controls by real-time PCR.**

***Results*: The *IRF5* rs2004640 (G/T) polymorphism did not confer risk of SLE or influence clinical or serological phenotype. However, the mutant allele conferred a borderline risk to develop thrombocytopenia (odds ratio: 2.05, 95% confidence interval: 0.97–4.3, *P*=0.06) in patients with SLE.**

***Interpretation & conclusions*: Our study revealed that the *IRF5* rs2004640 polymorphism was not a risk factor for SLE in population from south India. It may, however, be a useful genetic marker for thrombocytopenia in SLE patients. Although we could not demonstrate susceptibility toward lupus in the presence of *IRF5* rs2004640 (G/T) polymorphism, further exploration of the genetic variability of *IRF5* may help uncover its pathogenic role in Indian SLE patients.**

Key words Autoantibodies - interferon-alpha - interferon regulatory factor 5 - polymorphism - systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by production of pathogenic autoantibodies against nuclear antigens. The aetiology of SLE is partly understood; however, genetic and environmental factors play a key role in determining the susceptibility, course and outcome of the disease. Higher incidence of disease within the family members supports the pivotal part of genetics with the pathogenesis of SLE. These aberrant genetic risk factors have a complex interplay and facilitate

the development of autoimmunity by disrupting (*i*) immune cell signalling, (*ii*) immune complex disposal, and (*iii*) augmenting the type I interferon synthesis[1]. Advances in human genetics and gene expression studies have helped in understanding the immunopathogenesis of SLE[2]. Cytokines are the major transducers of immune signals and play a major role in autoimmune pathogenesis. One such cytokine is interferon alpha (IFN-α), a pleiotropic type I IFN with the potential to break immune tolerance, activation of autoreactive T and B cells and amplifying the autoimmune response. It was reported that interferon therapy augmented the secretion of autoantibodies in patients treated with IFN-α for non-autoimmune disorders[3,4]. The elevated serum IFN-α level corresponds with an elevated 'IFN-α signature' in peripheral blood mononuclear cells in lupus patients[2,5].

The major pathogenic mechanism associated with the elevated secretion of IFN-α in SLE patient is as follows. Toll-like receptors (TLRs) and other pattern-recognition receptors present in the dendritic cells, upon recognizing the specific ligand and/or antigens in immune complexes enhance the secretion of IFN-α through the interferon regulatory factor 5 (IRF5) transcription system[2]. IRF5 is a transcription factor which induces the transcription of pro-inflammatory cytokines such as IFN-α, tumour necrosis factor-α (TNF-α), interleukin 12 (IL)-12 and IL-6 through MyD88-dependent activation of nuclear factor-κB (NF-κB) pathway[6]. The *IRF5* polymorphisms have also been shown to be a genetic risk factor for other autoimmune diseases such as SLE[7], scleroderma[8], inflammatory bowel disease[9], Sjögren's syndrome[10] and rheumatoid arthritis[11]. Richez *et al*[6] described the details of the variants in *IRF5* gene, the molecular mechanism by which these genetic variants augment the secretion of IFN-α and mutant allele frequency in susceptible populations. One such genetic variant in *IRF5* gene was the rs2004640 G/T polymorphism reported to be a genetic risk factor for SLE in Caucasian, Afro-American and in Asian ethnic population[12-18]. A recent study of three polymorphisms (rs10954213, rs2004640 and rs2280714) in *IRF5* gene revealed that the GTA haplotype was a risk for SLE and the rs2004640 T allele was an independent risk factor for SLE and development of lupus nephritis in Egyptian children[19]. The rs2004640 G/T polymorphism was reported to alter the splicing process and lead to excessive production of IFN-α, which might augment the autoimmune responses through its pleiotropic effects on various immune cells[6,7]. Identifying the genetic modifications of critical molecules in the type I IFN pathway is expected to increase the understanding of disease pathogenesis and its impact on patients with SLE. This study was carried out to determine the frequency of *IRF5* rs2004640 polymorphism in SLE patients and healthy controls among south Indian population and to analyze its influence on pathogenesis, clinical and autoantibody profile of lupus.

Material & Methods

This study was carried out in the Clinical Immunology OPD at Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry, India, during September 2009 to March 2012. The sample size for cases and controls was calculated using CaTS power calculator for genetic studies[20] with a power of 0.8 and 0.05 as the significance level. A total of 300 SLE patients attending Clinical Immunology OPD, fulfilling the 1997 American College of Rheumatology criteria for SLE[21] were included as cases. The disease activity in SLE patients was assessed and graded by systemic lupus erythematosus disease activity index[22]. A total of 460 age- and sex-matched volunteers with no known family history of autoimmune diseases, diabetes mellitus, malignancies hypertension and other co-morbidities were included as control population. The Institute Ethics Committee of JIPMER had approved the study. A signed written informed consent was obtained from all the participants.

Peripheral venous blood (5 ml) was collected from the participants and was used to extract genomic DNA by phenol-chloroform method following the published protocol[23]. This protocol involved selective osmotic lysis of RBCs, removal of haemoglobin by repeated washing and concentration of leucocytes. The DNA from the concentrated leucocytes was obtained by lysing the cells using hypertonic saline and proteinase K enzyme. Phenol-chloroform was added to localize the DNA in aqueous phase; later, the DNA was precipitated in absolute ethanol and dissolved in Tris EDTA buffer (*p*H 8.0). The DNA concentration was measured (Picodrop, Thermo Scientific, USA) and diluted to contain 50 ng/µl. The diluted DNA was used for genotyping protocols. The *IRF5* gene rs2004640 G/T polymorphism was tested by TaqMan real-time genotyping assay using the primers and probes obtained from Applied Biosystems (CA, USA). The primer pairs used for IRF5 G/T genotyping were

Forward-5'-CAGCTGCGCCTGGAAAG-3' and Reverse 5'-GGGAGGCGCTTTGGAAGT-3'. The probe sequences used for detection of G and T alleles were VIC: TGTAGGCACCCCCCCG and FAM: TGTAGGCACCCACCCG, respectively.

Statistical analysis: The genotype and allele frequencies between controls and cases were compared using the Chi-square test with Yates's correction or Fisher's exact test. Relative risk conferred by the mutant allele was arrived by calculating the odds ratio (OR) and confidence interval 95 per cent (CI 95%). Age, gender, complement level, disease duration, organs involved, autoantibodies and *IRF5* genotypes were used as covariates to perform the logistic regression analysis. Statistical analysis for this study was carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Table I. Demographic details of the study participants

Demographic features	Patients (n=300)	Controls (n=460)
Females	279	420
Males	21	40
Male:female	1:13	1:11
Age (yr) (mean±SD)	27.9±9.6	33±12
Age at onset (yr) (mean±SD)	25.5±8.4	-
Mean disease duration (months) (mean±SD)	72.5±40	-

Results

The demographic features of the cases and controls are depicted in Table I. Major clinical manifestations and the autoantibody reactivity are presented in Table II. The *IRF5* rs2004640 C/T genotype and allele distribution amongst cases and controls are given in Table III. It was observed that the distribution of minor allele T was almost similar in patients (32%) and controls (30%). The homozygous and the heterozygous mutant genotypes were found to be almost equally distributed in both the cases and controls and thus did not reveal any significant risk to develop SLE. Analysis of influence of *IRF5* rs2004640 G/T polymorphism on clinical phenotypes revealed a borderline association with the development of immune thrombocytopenia (ITP) in patients with homozygous mutant genotype (P=0.06, OR - 2.05, 95% CI=0.97–4.3) (Table IV).

Discussion

SLE is a multisystem autoimmune disorder characterized by the production of autoantibodies with an overwhelming immune response against self-antigens. A severe deviation from immune homeostasis is the classical feature of SLE which includes a compromised cytokine profile favouring only the amplification of the autoimmune response. An increased circulatory level of IFN-α is a prominent finding in active SLE and its rise in the serum is directly proportional to severity of the disease[1,2,24]. In our control population, a 30 per cent prevalence of the minor allele

Table II. Clinical and autoantibody phenotypes in systemic lupus erythematosus patients

Clinical manifestations	Number of patients (%) (n=300)	Autoantibody profile	Number of patients (%)
Mucocutaneous disease	247 (82)	ANA	273 (91)
Joint involvement	210 (70)	Anti dsDNA	211 (70)
Haematological disorders	146 (49)	Anti-nucleosome	138 (46)
Nephritis	131 (44)	Anti Ro/SSA	126 (42)
CNS lupus	87 (29)	APLA (ACL, β2GPI, LAC)	126 (42)
Vasculitis	82 (28)	Anti SSB/La	40 (13)
Serositis	51 (17)	Anti Sm	106 (35)
Secondary APS	36 (12)	Anti U1RNP	98 (33)
Mean C3 (g/l) (mean±SD)	0.725±0.65	Anti Ro52	92 (31)
Mean C4 (g/l) (mean±SD)	0.231±0.1028	Anti-histone	86 (29)
Mean SLEDAI	14.61±7.92	Anti-ribosomal P	78 (26)

SLEDAI, systemic lupus erythematosus disease activity index; CNS, central nervous system; APS, antiphospholipid syndrome; SD, standard deviation; ANA, antinuclear antibody; Anti dsDNA, anti double stranded DNA antibody; Anti Ro/SSA, anti Ro/ Sjogren syndrome A antibody; APLA, antiphospholipid antibody; ACL, anticardiolipin antibody; β2GPI, anti beta2 glycoprotein 1 antibody; LAC, lupus anticoagulant antibody; Anti SSB/La, anti- Sjogren syndrome antigen B/La antibody; Anti Sm, anti-Smith antibody; Anti-UIRNP, anti-U1 ribonucleoprotein antibody; Anti-Ro52, anti Ro 52 kDa antibody

Table III. Genotype distribution of *IRF5* G/T (rs2004640) polymorphism in patients and controls

IRF5 G/T genotype	Patients n=300 (%)	Controls n=460 (%)	*P*	OR	95% CI
GG	143 (48)	235 (51)			
GT	121 (40)	172 (38)	0.38	1.15	0.84-1.6
TT	36 (12)	53 (11)	0.71	1.16	0.7-1.8
GG versus GT + TT	157	225	0.3	1.14	0.9-1.53
GG + GT versus TT	264	407	0.9	1.00	0.6-1.5
Alleles (%)					
G	407 (68)	642 (70)			
T	193 (32)	278 (30)	0.4	1.1	0.9-1.4

Age, sex and family history of autoimmune diseases were considered as confounding variables to derive the adjusted odds ratio.
OR, odds ratio; CI, confidence interval

Table IV. Influence of *IRF5* G/T (rs2004640) polymorphism on various haematological manifestations of systemic lupus erythematosus

Clinical manifestations	*IRF5* genotype			*P*	OR	95% CI
	Genotype	Number of positives (%)	Number of negatives (%)			
Lymphopenia (n=90)	GG	41 (46)	102 (49)			
	GT	39 (43)	82 (39)	0.52	0.8	0.49-1.43
	TT	10 (11)	26 (12)	0.9	1.04	0.46-2.36
	GG versus GT + TT	49	108	0.7	1.129	0.7-1.85
	GG + GT versus TT	80	184	0.84	1.13	0.52-2.45
ITP (n=70)	GG	28 (40)	115 (50)			
	GT	29 (41)	92 (40)	0.88	1.06	0.6-1.82
	TT	13 (19)	23 (10)	0.06	2.053	0.97-4.3
	GG versus GT + TT	42	115	0.17	1.5	0.87-2.58
	GG + GT versus TT	57	207	0.06	0.487	0.23-1.02
AIHA (n=59)	GG	30 (51)	113 (47)			
	GT	19 (32)	102 (42)	0.18	0.64	0.35-1.18
	TT	10 (17)	26 (11)	0.17	1.68	0.76-3.72
	GG versus GT + TT	29	128	0.66	0.85	0.48-1.5
	GG + GT versus TT	49	215	0.6	0.86	0.51-1.42
Neutropenia (n=22)	GG	8 (36)	135 (49)			
	GT	11 (50)	110 (40)	0.37	1.52	0.6-3.6
	TT	3 (14)	33 (11)	0.73	1.17	0.3-4.179
	GG versus GT + TT	14	143	0.37	1.65	0.67-4.06
	GG + GT versus TT	19	245	0.73	0.73	0.23-3.041

Age, sex and family history of autoimmune diseases were considered as confounding variables to derive the adjusted odds ratio.
ITP, immune-mediated thrombocytopenia; AIHA, autoimmune haemolytic anaemia; OR, odds ratio; CI, confidence interval

frequency was observed, which was similar to that reported from China[12], Japan[13] and Korea[14] but lesser than the reports of 50 per cent in Caucasians[16-18,25] and African Americans[26].

In this study, a marginally higher frequency of the mutant allele was observed in SLE patients in comparison to the controls (32 vs 30%); however, the difference was not significant. A similar negative association between the variant T allele with SLE was reported in the Chinese[12] and Japanese[13] but not in Koreans[14] and a subgroup of Shandong Han Chinese population[15]. Although a significant association was

observed in Koreans, the reported odds ratio was 1.32, which rendered their results ambiguous. In addition to the above SNP, analysis of three more variants, namely, the rs729302, rs752637 and rs2280714 did not appear to confer a significant risk[14]. The haplotypes constructed from these polymorphisms also failed to show a positive association with the development of SLE in Koreans[14]. In Caucasians, the *IRF5* 2004640 T allele was reported to confer a significant risk to develop SLE[16-18,25], which was also replicated in many of the genome wide association studies (GWAS) studies conducted in Caucasians[27]. Kawasaki *et al*[13] analyzed the *IRF5* variants such as rs2004640, rs10954213, rs6953165, rs41298401 and rs11770589 in a Japanese cohort and found that none of these were associated with SLE susceptibility. However, when they combined their results with a Korean cohort, they noted a significant association with the development of SLE in Asians. They concluded that the polymorphism in Intron 1 of *IRF5* gene played a crucial role in the expression of IFN pathway genes. Dang *et al*[28] reported that the *IRF5* 2004640 polymorphism was not a risk for SLE in northern Han Chinese. They observed significant interaction and a higher incidence of mutant alleles of *IRF5* (rs2004640) and STAT4 (rs7574865) variants in SLE patients.

In our study the patients with *IRF5* rs2004640 mutant allele T showed a trendency to develop ITP. It was observed that a larger proportion of SLE patients manifesting ITP were carriers of *IRF5* homozygous mutant genotype (19 vs 10%) (data not shown). Stratification of patients based on the clinical phenotype might have rendered the sample size low to obtain a significant association. Wazny and Ariano[29] reported that the major side effect of IFN-α therapy was thrombocytopenia attributable to bone marrow suppression, immune-mediated destruction and platelet aggregation. Therefore, ITP in SLE patients might be due to the elevated secretion of IFN-α under the influence of *IRF5* rs2004640 mutant allele.

Although the *IRF5* rs2004640T variant allele was reported to be associated with the development of lupus nephritis in Chinese SLE patients[28], we did not find any such association in our patients. The frequency of the anti-dsDNA and anti-Ro 52 antibodies with TT genotype was higher in patients versus controls, *i.e*. 14 per cent versus 9 per cent and 18 per cent versus 10 per cent, respectively; however, the difference was not significant which might be due to a small sample size (data not shown). Similar findings were also reported by Qin *et al*[30] in patients with lupus nephritis. Niewold *et al*[31] reported that four SNPs (rs2004640, rs3807306, rs10488631 and rs2280714) along with insertion polymorphisms in the promoter region and exon 6 of the *IRF5* gene influenced the production of autoantibodies in SLE patients and in their family members from European ancestry. They also reported a few haplotypes which appeared to augment the production of IFN-α and conferred risk to develop SLE. They highlighted the differential effect of *IRF5* genotype on serum IFNα activity that was detectable only in patients who were positive for either anti-ribonuclear protein antibodies (anti-Ro, La, Sm and RNP) or anti-dsDNA but was not seen in patients with higher background IFNα activity, who were positive for both autoantibodies. We did not observe any positive association between the rs2004640 mutant allele T and the production of autoantibodies (data not shown). It has been reported that the autoantigen-antibody complexes can themselves augment the IFN-α production through the endosomal TLR system. The plasmacytoid dendritic cells with the help of TLR 9 were capable of recognizing the dsDNA in immune complexes and trigger the production of IFN-α. The mechanism by which the IFN-α induces and amplifies the autoimmune responses has been described in detail[32].

The major limitation of our study was that we analyzed *IRF5* rs2004640 polymorphism only. Study of other major polymorphisms in the *IRF5* gene would further help to establish the population-specific risk haplotype. In various studies, *IRF5* pathway has been targeted by therapeutics directed at the endosomal TLRs and IFN-α[33-35]. Thus, *IRF5* genotype may help to differentiate between the responders and non-responder patients with respect to these therapies.

In conclusion, our study revealed that the *IRF5* rs2004640 polymorphism was not a risk factor for SLE in population from southern India. However, with a larger sample size, it may emerge as a possible genetic marker to predict the development of thrombocytopenia in SLE patients. Screening the other polymorphisms in *IRF5* gene will provide an insight into the role of *IRF5* genetics in SLE pathogenesis.

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***Conflicts of Interest*:** None.

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