Signal transducer and activator of transcription and the risk of rheumatoid arthritis and thyroid autoimmune disorders

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Abstract

Objective

The signal transducer and activator of transcription 4 (STAT4) gene localised on chromosome 2q32.2–q32.3 is known to be essential for mediating responses to interleukin 12 in lymphocytes and regulating the differentiation of T helper cells. The aim of this study was to investigate the role of the STAT4 gene in susceptibility to rheumatoid arthritis (RA) and autoimmune thyroid diseases (AITDs) in Tunisian case control studies.

Methods

Genotyping of STAT4 rs7574865 single nucleotide polymorphism (SNP) was performed in 140 patients affected with RA, 159 patients affected with AITDs and 200 healthy controls using TaqMan® allelic discrimination assay. Data were analysed by χ^2-test, genotype relative risk (GRR) and odds ratio (OR).

Results

Our results revealed that frequencies of the T allele and the T/T genotype were significantly higher among RA patients compared to controls (p=0.008; p=0.003, respectively). However, no significant associations with the risk of autoimmune thyroid diseases were detected. Moreover, the stratification of RA patients subgroups revealed a significant association of both T allele and T/T genotype in patients presented erosion (p=0.003; p=0.004, respectively) as well as anti-cyclic peptides-negative RA (ACPA⁻) (p=0.002; p=0.0003, respectively). Furthermore, genotypic association was found according to the absence of rheumatoid factor antibody (RF) (p=0.0014). But, no significant differences in allele and genotype frequencies of STAT4 rs7574865 polymorphism were detected according to the presence of another autoimmune disease, nodules and in HLA-DRB1*04 and HLA-DRB1*0404 positive subgroups.

Conclusion

Our results support involvement of the STAT4 gene in the genetic susceptibility to RA but not to AITDs in the Tunisian population.

Key words

STAT4 gene, rheumatoid arthritis, autoimmune thyroid diseases, association study

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Introduction

Autoimmune diseases (AIDs) are multifactorial diseases and considered to be caused by interactions of both genetic and environmental risk factors such as tobacco smoking, hormones, diet, drugs, toxins and/or infections (1). They are characterised by the loss of immunological tolerance to self antigens and multiple alterations in the immune system resulting in a spectrum of syndromes that either target specific organs or affect the body systemically. Despite their relatively high prevalence rate, the etiology and pathogenesis of most AIDs remain poorly understood (2). Several susceptibility genes have been identified as common risk factors for different autoimmune diseases. For examples, *PTPN22* has been reported as a susceptibility gene for rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Graves' disease (GD), type 1 diabetes (T1D) and juvenile idiopathic arthritis (JIA) (3). *CTLA4* and *FCRL3* also were reported to be associated with several autoimmune diseases such as RA, SLE and autoimmune thyroid diseases (AITDs) (4, 5). Similar to these susceptibility genes, *STAT4* (Signal Transducer and Activator of Transcription protein 4) is also associated with risk for RA, SLE, primary Sjögren's syndrome (pSS), inflammatory bowel diseases (IBD) and T1D (6-10). Aiming to define whether this gene plays a key role in multiple autoimmune diseases, we investigated the association of the most significant disease-associated SNP (rs7574865) located within the third intron of the *STAT4* gene at chromosome 2q32.2-q32.3 (6, 7, 11), with rheumatoid arthritis (RA) as a non-organ specific and AITDs as an organ specific autoimmune disease in the Tunisian population.

STATs are a family of latent cytoplasmic transcription factors. Upon cytokine stimulation, STATs become tyrosine phosphorylated and translocate into the nucleus where they bind to DNA to activate transcription (12). *STAT4* is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of inflammation in human beings (13). *STAT4* is required for Th1 cell differentiation and proliferation, is activated by IL-12, and induces the transcription of IFN-γ (14, 15). In addition, STAT4 is implicated in the optimal differentiation of a newly defined CD4^{+} T-cell lineage, designated Th17 cells. Dependent in part on the activity of interleukin-23, a cytokine related to interleukin-12 (16), proinflammatory Th17 cells can play an important, if not predominant, role in chronic inflammatory disorders (17).

Patients and methods

RA sample

Our study included 140 Tunisian RA patients (25 men and 115 women). The mean ages were 39.3 years at disease onset and 62 at the time of this study. Among our RA patients, 98 had radiographically apparent hand erosions, 15 presented rheumatoid nodules; 96 were rheumatoid factor positive (RF[+]); 92 were seropositive for ACPA; 57 had another autoimmune disease (Sjögren's syndrome, AITDs, SLE and T1D), 58 were positive for HLA-DRB1*04 and 10 were positive for HLA-DRB1*0404. All cases satisfied the 1987 American College of Rheumatology (ACR) criteria for RA (18).

AITD sample

One hundred and fifty-nine unrelated Tunisian patients affected with AITDs were studied (118 were women and 41 were men). There were a total of 99 patients with Graves' disease (GD) and 60 patients with Hashimoto's thyroiditis (HT). The mean age of the disease onset was 34.2 years. The diagnosis of GD was based on the presence of biochemical hyperthyroidism as indicated by a decrease of TSH, an increase of T4 levels and positive TSH receptor antibody, in association with diffuse goiter or the presence of exophthalmoses. The diagnosis of HT was based on the presence of thyroid hormone replaced primary hypothyroidism, defined as a TSH level above the upper limits associated with positive titters of thyroid autoantibodies (antithyroglobulin and/or anti-thyroid peroxydase) and with or without a palpable goiter.

Control group

We used two hundred ethnically matched healthy controls (60 men and

140 women) without clinical evidence of any autoimmune diseases, based on medical history and physical examination. The mean age at analysis was 43.2 years.

All individuals (patients and controls) provided informed consent as required by the ethics committee of the Centre Hospitalo-Universitaire Hédi Chaker de Sfax, Tunisie.

Autoantibody analysis

RA Patient sera obtained at the time of diagnosis were examined for ACPA by enzyme-linked immunosorbent assay (ELISA) (Anti-CCP ELISA (IgG) Test instruction; EUROIMMUN®, Allemagne) and for RF by nephelometry. For AITDs patients' thyroid autoantibodies (anti-thyroglobulin and anti-thyroid peroxydase) were measured by enzyme-linked immunosorbant assay (ELISA) and indirect immuno-fluorescence using commercially available kits (BINDAZYMETM Human EIA kits) with the respective normal ranges of 0 to 100 and 0 to 70 IU/ml.

HLA-DR typing

Broad-level HLA-DRB1 typing and high-resolution DRB1*04 typing were accomplished by polymerase chain reaction-sequence specific primers (PCR-SSP) (all DRB1 alleles for broad-level typing, and group-specific amplification for DRB1*04 alleles) using commercially available kits (Micro SSP™ DNA Typing trays, SSP2LB and SSP2-104; respectively) (One Lambda Inc, USA).

Molecular genotyping

Genomic DNA was isolated and purified from fresh peripheral blood leucocytes according to standard protocols. Genotyping of the *STAT4* rs7574865 SNP was carried out with a TaqMan 5' allelic discrimination assay on an *ABI 7500* real-time polymerase chain reaction (PCR) machine (assay: C_29882391_10, Applied Biosystems, Foster City, CA, USA) according to manufactor's instructions. CEPH controls (1347-02 and 884-15) were co-genotyped with all our samples for quality control. Moreover, ten percent of randomly chosen samples were genotyped twice.

Table I. Allele and genotype frequencies of the *STAT4* G/T polymorphism analysed in RA patients and healthy controls.

	RA group n=140 (%)	Control group n=200 (%)	*p*-value	OR (95% CI)
Allele				
G	210 (75)	333 (83.2)		
T	70 (25)	67 (16.8)	0.008	1.66 (1.14-2.42)
Genotype				
G/G	81 (57.8)	135 (67.5)		
G/T	48 (34.3)	63 (31.5)		
T/T	11 (7.9)	2 (1)		
T/T *vs.* (G/G+G/T)	129 (92.1)	198 (99)	0.003	8.44 (1.84-38.71)

(%): Frequencies of alleles; (*): Significant *p*-value (*p*<0.05); OR: Odds ratios and 95%; CI: confidence intervals.

Statistical analyses

Hardy-Weinberg equilibrium was checked in the control group using a standard chi-square test. Genotypic and allelic frequencies were obtained by direct counting. The χ^2 test (2*2 contingency tables) with 1 degree of freedom was performed to compare distributions for statistical significance. The Genotype Relative Risk (GRR) test adjusts the genotype frequencies in the controls to the expected Hardy-Weinberg proportions and yields more accurate risk estimates (19). Values of *p*<0.05 were considered statistically significant, but for RA subgroups, Significance of *p*-values was assessed using a Bonferroni correction at 5% (a *p*-value less than 0.05/11=0.0045 is considered significant). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated according to Woolf's method (20).

Power calculation

We calculated the empirical power of the study by bootstrapping from the dataset of patients and controls (random sampling with replacement of the same number of patients and controls from the original dataset). For each bootstrap a GRR test was calculated. 100 000 bootstraps were used and the empirical power was estimated as the number of times the GRR test exceeded the χ^2 threshold (when a true association is declared) at a 5% significance level (3.84) divided by the total number of bootstraps. Calculations were performed using a program written in R language (www.r-project.org) (21).

Results

We analysed 499 samples (140 RA patients, 159 patients affected with AITDs and 200 healthy controls). The distribution of genotypes in the control group showed no deviation from Hardy-Weinberg equilibrium (*p*=0.067).

Association of STAT4 polymorphism with RA

Table I show the *STAT4* rs7574865 allele and genotype distributions. We observed a statistically significant increase of the minor T allele in RA patients compared to healthy controls (25 *vs.* 16.8%, *p*=0.008, OR=1.66, 95% CI=[1.14–2.42]). Moreover, the distribution of genotypes showed a higher frequency of T/T homozygous individuals in the RA patient group (7.9 *vs.* 1% in the control group, *p*=0.003, OR=8.44, 95% CI= [1.84–38.71]).

Stratification by autoantibody status

When the subgroups defined by either ACPA positivity or RF positivity were compared with controls, no association could be observed for the *STAT4* polymorphism (Table II). Moreover neither allelic nor genotypic association was found in the subgroup who was positive for both tests (ACPA+ RF+) (*p*=0.23; *p*=0.67, respectively). However, a significant association of T allele and T/T genotype were shown with ACPA-negative RA in patients as compared with healthy controls (*p*=0.002; *p*=0.0003, respectively) (allelic OR=2.15, 95% CI=[1.3–3.55]; genotypic OR=14.14, 95% CI=[2.76–72.52]). Furthermore, an increase in

Table II. Allele and genotype frequencies of the *STAT4* rs7574865 SNP in Tunisian RA patients and Tunisian controls, stratified by clinical and immunological data.

	Allele association				Genotype association				
	G	T	*p*-value	Allelic OR (95% CI)	G/G	G/T	T/T	T/T *vs.* (G/G+G/T) *p*-value	Genotypic OR (95% CI)
ACPA[+] (n=92) [a]	143 (77.7%)	41 (22.3%)	0.1		56 (61%)	31 (33.6%)	5 (5.4%)	0.105	
ACPA[-] (n=48) [a]	67 (69.8%)	29 (30.2%)	0.002*	2.15 [1.3–3.55]	25 (52.1%)	17 (35.4%)	6 (12.5%)	0.0003*	14.14 [2.76–72.52]
RF[+] (n=96) [b]	144 (75%)	48 (25%)	0.015		54 (56.25%)	36 (37.5%)	6 (6.25%)	0.044	
RF[-] (n=44) [b]	66 (75%)	22 (25%)	0.06		27 (61.37%)	12 (27.27%)	5 (11.36%)	0.0014*	12.69 [2.38–67.78]
ACPA[+] RF[+] (n=68) [c]	107	29	0.23		41	25	2	0.67	
ACPA[-] RF[-] (n=21) [c]	31	11	0.12		12	7	2	0.023	
AID[+] (n=57)	84 (73.7%)	30 (26.3%)	0.021		29 (50.9%)	26 (45.6%)	2 (3.5%)	0.483	
Erosive [+] (n=98)	143 (73%)	53 (27%)	0.003*	1.84 [1.22–2.77]	53 (54.1%)	37 (37.7%)	8 (8.2%)	0.004*	8.8 [1.83–42.27]
Nodules[+] (n=15)	22 (73.3%)	8 (26.7%)	0.16		9 (60%)	4 (26.7%)	2 (13.3%)	0.005	
HLA-DRB1*04 (n=58)	91 (78.4%)	25 (21.6%)	0.23		34 (58.6%)	23 (39.7%)	1 (1.7%)	0.893	
HLA-DRB1*0404 (n=10)	13 (65%)	7 (35%)	0.036		4 (40%)	5 (50%)	1 (10%)	0.035	
Controls (n=200)	333 (83.2%)	67 (16.8%)	–		135 (67.5%)	63 (31.5%)	2 (1%)	–	

ACPA: anti-citrullinated peptide antibodies; RF: rheumatoid factor; AID: autoimmune diseases; (%): Frequencies of alleles; (*): Significant *p*-value using a Bonferroni correction at 5% (≤0.004); OR: Odds ratios and 95%; CI: confidence interval; [a] A direct comparison of ACPA[+] *vs.* ACPA[-] samples showed no significant difference; [b] A direct comparison of RF[+] *vs.* RF[-] samples showed no significant difference; [c] A direct comparaison ACPA[+] RF[+] *vs.* ACPA[-] RF[-] showed no significant difference.

frequency of the T/T genotype was observed in patients with RF-negative RA as compared with healthy controls (*p*=0.0014, genotypic OR=12.69, 95% CI=[2.38–67.78]). But, no significant association was found when we compared the ACPA–negative and RF-negative patients (ACPA[-] RF[-]) with controls (*p*>0.004).

In order to investigate the effect of serum autoantibodies on the association of *STAT4* with RA, we analysed the distribution of the rs7574865 genotypes and alleles among RA patients stratified according to autoantibody status. We found a similar trend of distribution between ACPA-positive and ACPA-negative patient groups and between RF-positive and RF-negative patient groups. Similarly, the comparison of the subgroups positive and negative for both ACPA and RF (ACPA[+] RF[+] *vs.* ACPA[-] RF[-]) showed no significant difference. This finding suggests that the susceptibility to RA conferred by the rs7574865 T allele does not differ according to ACPA or RF status in patients with RA (Table II).

Stratification by clinical data in RA patients

The stratification of RA patients' subgroups according to clinical data (Table II) revealed a significant associa-tion of both T allele and T/T genotype in patients presented erosion (*p*=0.003; *p*=0.004, respectively). But, no significant differences in allele and genotype frequencies of *STAT4* rs7574865 polymorphism were detected neither according to the presence of another autoimmune disease and nodules nor in HLA-DRB1*04 and HLA-DRB1*0404 positive subgroups (*p*>0.004).

Association of STAT4 polymorphism with AITDs

The data summarised in Table III show the allele frequency and genotype distribution in AITDs patients and controls. Our results showed neither allelic nor genotypic significant association of the *STAT4* gene polymorphism between AITDs patients and controls in the Tunisian sample (*p*=0.244; *p*=0.214, respectively); [OR$_{(95\% CI)}$=1.25 (0.86–1.83)]. Stratifying patients affected with AITDs according to their phenotype (Graves' disease and Hashimoto's thyroiditis) did not show any significant association with *STAT4* rs7574865 (data not shown).

Power calculation

To evaluate the informativeness of our case-control RA sample, we computed

Table III. Allele and genotype frequencies of the STAT4 G/T polymorphism analysed in AITD patients and healthy control subjects.

	AITDs group n=159 (%)	Control group n=200 (%)	*p*-value	OR (95% CI)
Allele				
G	254 (80)	333 (83.2)		
T	64 (20)	67 (16.8)	0.244	1.25 (0.86-1.83)
Genotype				
G/G	102 (64)	135 (67.5)		
G/T	50 (32)	63 (31.5)		
T/T	7 (4)	2 (1)		
T/T *vs.* (G/G+G/T)	152 (96)	198 (99)	0.214	4.56 (0.93-22.26)

(%): Frequencies of alleles; (*): Significant *p*-value (*p*<0.05); OR: Odds ratios and 95%; CI: confidence intervals.

the empirical power, which is the probability of detecting an association. We had a 77.8% power to detect a trend in favour of an association for α=5% for the allelic test and 98.3% power for the genotypic test. Likewise, for the AITD sample, we found a power of 23.9% for the allelic test and 77.4% for the genotypic test; this explains the negative allelic and genotypic associations.

Discussion

Our results suggest that *STAT4* rs7574865 T allele and T/T genotype were significantly associated with RA in the Tunisian population (*p*=0.008; *p*=0.003, respectively). These results support previous finding reported in US (6), Swedish (6, 22), Korean (7), UK (23), Greek (24), Colombian (25), Spanish (22), Japanese (26) and Dutch (22) populations. These results confirm that the *STAT4* rs7574865 polymorphism is associated with RA susceptibility in different ethnic groups, and that its prevalence is ethnicity dependent, as reported by Lee *et al*. (27). Moreover, in one meta-analysis, an association between the *STAT4* rs7574865 polymorphism and RA in all this studies, was shown (*p*<0.001, OR=1.271, 95% CI=1.197–1.350) (27). After stratification by ethnicity, analysis indicated that the *STAT4* rs7574865 T allele was significantly associated with RA in Europeans and Asians (*p*<0.001, OR=1.300, 95% CI=1.195–1.414; *p*<0.001, OR=1.216, 95% CI=1.135–1.303).

It is worth noting that the North American Rheumatoid Arthritis Consortium (NARAC) has reported an RA linkage region on the long arm of chromosome 2 in 642 families of European ancestry (28). In addition, dense SNP mapping and candidate-gene approach of this region led to the identification of a new susceptibility gene, *STAT4*, for RA and SLE (6). In fact, association of rs7574865 polymorphism was found in three SLE cohorts (6, 25, 26), suggesting that this locus may harbour a risk allele pre-disposing to susceptibility for multiple autoimmune diseases with a shared aetiology in a similar manner to the *PTPN22* gene.

In RA, previous studies have suggested that genetic risk factors predispose individuals to specific subsets of the disease, characterised by autoantibody status. For example, both the *HLA* shared epitope and *PTPN22* loci have been shown to be associated with a clear predisposition to ACPA-positive disease only. In our study, stratification of patients according to autoantibodies status showed a significant association of T allele with ACPA-negative RA subgroup and an increase of the T/T genotype in both ACPA-negative and RF-negative RA as compared with healthy controls. In this term, significant associations between the T allele and RA in both ACPA positive and negative RA patients *versus* controls were shown in a Caucasian population (29) and only with ACPA positive RA in a Korean population (7). Additionally, direct comparison between ACPA positive and negative subjects showed no significant difference in our study similar to the other previous studies (29). However, the association between the *STAT4* rs7574865 polymorphism and RA is not dependant of the presence of this antibody. It was also reported in two series of Spanish patients with RA that there was a lack of association between *STAT4* and RA with or without cardiovascular events, the presence of endothelial dysfunction or increased carotid intima-media thickness (30), or the presence of HLA shared epitope, rheumatoid nodules and radiographic changes (31).

In contrast to our positive results on the RA subgroup, we failed to detect either allelic or genotypic association of *STAT4* rs7574865 with AITDs and healthy controls in the Tunisian population. To assess the significance of our case-control sample, we computed the power of detecting an association; we found a power of 23.9% for the allelic test and 77.4% for the genotypic test. Therefore, the lack of association between *STAT4* polymorphism and AITDs could be due to the limited sample size. Our results were similar to a previous study that showed a lack of association of rs7574865 polymorphism with multiple sclerosis in the Spanish population (9).

In our study, we noted that the minor allele frequencies (MAF) in our healthy controls (16.8%) was similar to that reported previously in the European pop-

ulation (16.5 to 23.16%) and lower than those reported in the Asian (30.97 to 33.8%) and Latin American (31.46%) populations (27, 32). This similarity in the allele frequencies in our population as compared to other European populations could be explained by the exchange between the North African populations and European population (33, 34). These data support our previous results on 103 microsatellite markers which showed a similarity between allele frequencies between Tunisian and CEPH populations (35).

In conclusion, this study investigated the association of *STAT4* with RA in the Tunisian population. Our results were the first to show an association of this genetic variant in a population of North African origin. There was no clear effect observed in patients with AITD. Replication studies using larger sample sizes and/or other ethnic populations will be required to confirm and establish any genetic association.

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