ARTICLE

HLA-DRB1, IRF5, and CD28 gene polymorphisms in Egyptian patients with rheumatoid arthritis: susceptibility and disease activity

Nora M. Said [1] · Nillie Ezzeldin[2] · Dina Said[2] · Amany M. Ebaid[2] · Dina M. Atef [1] · Rehab M. Atef[1]

Abstract

This study was established to assess the effects of IRF5 rs10488631 and CD28 rs1980422 single-nucleotide polymorphisms (SNPs) and HLA-DRB1 shared epitope (SE) allele on the prognosis and disease activity of rheumatoid arthritis (RA) patients. A total of 150 RA patients and 150 healthy controls were genotyped for the selected SNPs by real-time PCR. HLA-DRB1 SE was determined using LAB Type SSO Class II DRB1 typing. Our results suggest that HLA-DRB1, CD28, and IRF5 significantly discriminated ($p < 0.001$) RA patients and healthy controls (OR of single HLA-DRB1 SE allele = 2.431, CI = 1.467–4.027, OR of two SE alleles = 11.152, CI = 2.479–50.159), (OR of CD28 risk allele C = 2.794, 95% CI = 1.973–3.956) and (OR of IRF5 risk allele C = 4.925, CI = 3.26–7.439). Rheumatoid factor (RF) seropositivity was associated with HLA-DRB1 SE ($p < 0.001$) and IRF5 risk allele ($p < 0.001$). ACPA was significantly associated only with IRF5 risk allele ($p < 0.001$). A better response to methotrexate therapy was found in HLA-DRB1 SE non-carriers, and CD28 TT patients. This study demonstrated associations of HLA-DRB1 SE, CD28, and IRF5 with the risk of RA. HLA-DRB1 SE and CD28 rs1980422 can be used as predictors of methotrexate therapy response.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease, causing destruction of cartilage and bone. Multiple factors influence its onset and progression, including genetic and environmental factors [1]. Genetic associations account for 40–60% of the total risk of developing RA. Human leukocyte antigen (HLA) genes, mainly at the HLA-DRB1 locus, have been most strongly confirmed to be associated with RA [2]. RA-linked DRB1 alleles show conserved amino acid sequences at positions 70–74 in the DRB1 chain. These alleles were named shared epitope (SE) alleles [3]. In the Egyptian population, a high prevalence of HLA-DRB1 SE has been reported among RA patients. Homozygosity for SE alleles confers a higher risk

for RA than heterozygosity. This characteristic feature of SE alleles is referred to as the gene dose effect [4].

The destruction of joints in RA patients is mediated by leukocytes, mainly T cells, infiltrating into the joints. These infiltrates produce proinflammatory cytokines and chemokines, causing cartilage destruction and bone erosion [5]. T-cell activation requires two signals: the first is through the T-cell receptor and the second is mediated by CD28 [6]. Several studies have reported an association between single-nucleotide polymorphisms (SNPs) in the CD28 gene and increased susceptibility to RA [5, 7].

Dysregulated expression of the type 1 interferon (IFN) gene has been detected in leukocytes of patients with autoimmune disorders. Interferon regulatory factor 5 (IRF5) is a regulatory protein involved in the regulation of type 1 IFN gene transcription. Moreover, it is crucial for the production of other proinflammatory cytokines, including TNF-α, IL-12, and IL-16 [8]. The IRF5 gene is located on chromosome 7q32.1. Multiple SNPs of this gene have been linked to the development of autoimmune disorders [9, 10].

Previous studies have reported that SNPs in CD28 (rs1980422) and IRF5 (rs10488631) are linked to the seropositivity and severity of RA [5, 7, 11]. Similarly, other studies have provided evidence for associations between HLA-DRB1 SE alleles and seropositivity and severity [3, 4].

✉ Nora M. Said
   dr.nora2014@yahoo.com

1   Department of Clinical Pathology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

2   Department of Rheumatology and Rehabilitation, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Therefore, we here investigated these associations in Egyptian RA patients and assessed the effects of these SNPs on the prognosis and disease activity.

Results

Demographics and laboratory results

The RA group ($n = 150$) consisted of 143 females (95.4%) and 7 males (4.6%), whereas the healthy controls ($n = 150$) consisted of 122 females (81.3%) and 28 males (18.6%). There were no significant differences between the studied groups in terms of age ($p > 0.05$). However, there was significant difference regarding sex ($p < 0.05$), so sex adjustment was done for genotype comparison. The results of laboratory investigations of the case group are shown in Table 1.

Table 1 Demographics and clinical characteristics of the studied groups.

Variables	Case (No. = 150)	Control (No. = 150)	p
Age (years)	46.4 ± 9.3	47.7 ± 8.08	0.8[a]
Sex			
Males	7 (4.6%)	28 (18.6%)	0.5[b]
Females	143 (95.4%)	122 (81.3%)	
ESR	50.9 ± 23.9		
CRP	14.8 ± 26.4		
RF	132.4 ± 174.2 6.4 (7–650)		
ACPA	183.8 ± 266.8 56.2 (7–1228.2)		
TLC	6.7 ± 2.5		
HB	11.1 ± 1.1		
PLT	258.8 ± 95.8		
Total bilirubin	0.4 ± 0.2		
Direct bilirubin	0.14 ± 0.1		
T.P	7.2 ± 0.6		
Albumin	3.6 ± 0.4		
Urea	20.7 ± 13.2 17.5 (11–67)		
Creatinine	0.6 ± 0.17		
ALT	23.1 ± 14.1 19.5 (1–73)		
AST	24.9 ± 10.9 22 (12–50)		
DAS 1st	5.1 ± 0.9		
DAS 2nd	4.8 ± 1.5		

Data were presented as mean ± SD, median and (range) or No. (%).
[a]t-Test.
[b]Chi square test.

Frequency of genotypes in studied groups

The distribution of HLA-DRB1 alleles among cases and controls was listed in Table 2 with alleles *01 and *04 showing highest frequency among cases (18% and 26.3% respectively).

All studied genes were in Hardy–Weinberg Equilibrium (Table 3). The differences of allele and genotype frequencies for *IRF5* (*rs10488631*), *CD28* (*rs1980422*), and *HLA-DRB1* between the studied groups are shown in Table 3. With regard to the *HLA-DRB1* SE allele, there was a significant increase in frequency of SE1 (carrying one SE allele) patients compared with the level in controls (45% vs. 28%, respectively, OR 2.431, CI = 1.467–4.027, $p < 0.001$) (Table 3). Moreover, the frequency of SE2 (carrying 2 SE alleles) patients was significantly higher than in controls (10.7% and 1.3%, respectively, OR 11.152, CI = 2.479–50.129, $p = 0.002$).

Regarding the *CD28* (*rs1980422*) allele, the frequency of the risk C allele was significantly higher among RA patients than in controls (52.0 vs. 27.0%, OR = 2.794, 95% CI = 1.973–3.956, $p < 0.001$). The frequencies of the risk genotypes CC (OR = 5.427, CI = 2.586–11.393) and CT (OR = 7.652, CI = 4.263–13.736) were significantly higher in RA patients (20% and 64%, respectively) than in healthy controls (12.8% and 28.6%, respectively) (both $p < 0.001$; Table 3).

With regard to the *IRF5* (*rs10488631*) allele, the results revealed a significant increase in the rate of the risk allele C (OR 4.925, CI = 3.260–7.439) in patients compared with that in controls (44.0% and 13.0%, respectively) ($p < 0.001$). Concerning the risk genotypes CC and CT, they were present at increased frequencies in cases compared with those in controls (28% vs. 4.7% and 32% vs. 16.7%, respectively). This increase was statistically significant regarding both genotypes (both $p < 0.001$), along with the increased frequency of the protective genotype TT in controls compared with that in cases (78.6% and 40%, respectively; Table 3).

Analysis of genotypes and clinical and laboratory findings

HLA-DRB1 SE was significantly associated only with positive rheumatoid factor (RF) ($p < 0.001$). The frequency of RF positivity was higher among SE1 and SE2 patients (86.8% and 81.3%, respectively) compared to SE0 patients. However, there was no significant difference between SE1 and SE2 groups. There was no significant association between *HLA-DRB1* SE and ACPA positivity ($p = 0.750$; Table 4).

The relationships of the laboratory findings with different *CD28* (*rs1980422*) alleles and genotypes in the RA patient

Table 2 Distribution of HLA-DR alleles among cases and controls.

HLA-DRB1	Cases (n = 300) No.	%	Control® (n = 300) No.	%	χ^2	p	OR	95% CI LL	UL
*01	54	18.0	13	4.3	28.24*	<0.001*	4.846	2.584	9.090
*03	32	10.7	38	12.7	0.582	0.445	0.823	0.499	1.358
*04	79	26.3	30	10.0	26.92*	<0.001*	3.217	2.038	5.078
*07	4	1.3	21	7.0	12.04*	0.001*	0.180	0.061	0.530
*08	6	2.0	10	3.3	1.027	0.311	0.592	0.212	1.650
*09	4	1.3	6	2.0	0.407	0.524	0.662	0.185	2.371
*10	1	0.3	1	0.3	0.0	1.000	1.000	0.062	16.063
*11	26	8.7	46	15.3	6.313*	0.012	0.524	0.315	0.873
*12	8	2.7	18	6.0	4.020*	0.045	0.429	0.184	1.003
*13	32	10.7	59	19.7	9.443*	0.002*	0.488	0.307	0.776
*14	0	0.0	12	4.0	12.25*	<0.001*	–	–	–
*15	34	11.3	35	11.7	0.016	0.898	0.968	0.586	1.598
*16	20	6.7	11	3.7	2.755	0.097	1.877	0.883	3.988

χ^2 chi square test, *OR* odds ratio, *CI* confidence interval, *LL* lower limit, *UL* upper limit, *p* p value for comparing between different categories.

*Statistically significant at $p \leq 0.0028$.

Table 3 Distribution of CD28, IRF5 genotyping and allele and HLA-DRB1 allele between the case and control groups.

	Cases (n = 150)	Control (n = 150)	χ^2	p	Crude OR OR	95% CI LL	UL	p	Adjusted by sex OR	95% CI LL	UL	HWE χ^2	p
HLA-DRB1 SE allele													
SE0®	66 (44.0%)	106 (70.7%)										0.006	0.940
SE1	68 (45.3%)	42 (28.0%)	14.789*	<0.001*	2.600	1.589	4.255	<0.001*	2.431	1.467	4.027		
SE2	16 (10.7%)	2 (1.3%)	16.951*	<0.001*	12.849	2.862	57.686	0.002	11.152	2.479	50.159		
CD28 rs1980422													
TT®	24 (16.0%)	88 (58.6%)										0.1255	0.723
CT	96 (64.0%)	43 (28.6%)	56.406*	<0.001*	8.186	4.597	14.579	<0.001*	7.652	4.263	13.736		
CC	30 (20.0%)	19 (12.8%)	24.218*	<0.001*	5.790	2.788	12.022	<0.001*	5.427	2.586	11.393		
Allele													
T®	144 (48.0%)	219 (73.0%)											
C	156 (52.0%)	81 (27.0%)	39.230*	<0.001*	2.930	2.083	4.119	<0.001*	2.794	1.973	3.956		
IRF5 rs10488631													
TT®	60 (40.0%)	118 (78.6%)										0.479	0.489
CT	48 (32.0%)	25 (16.7%)	21.687*	<0.001*	3.776	2.125	6.708	<0.001*	3.542	1.976	6.348		
CC	42 (28.0%)	7 (4.7%)	42.000*	<0.001*	11.80	5.001	27.841	<0.001*	10.863	4.567	25.843		
Allele													
T®	168 (56.0%)	261 (87.0%)											
C	132 (44.0%)	39 (13.0%)	70.740*	<0.001*	5.258	3.502	7.895	<0.001*	4.925	3.260	7.439		

χ^2 chi square test, *OR* odds ratio, *CI* confidence interval, *HWE* Hardy–Weinberg equilibrium, *LL* lower limit, *UL* upper limit, ® reference group, *p* p value for comparing between the studied groups.

*Statistically significant at $p \leq 0.0028$.

group are shown in Table 5. Our results revealed no statistically significant increase in RF and ACPA positivity in those neither with risk allele C, nor with risk genotypes CC and CT. However, regarding *IRF5* (*rs10488631*), there were statistically significant increases in the frequency of both RF and ACPA positivity in those with risk genotypes CC and CT vs. the protective genotype TT. The frequencies of RF positivity were 71.4% in CC patients, 87.5% in CT patients,

and 60% in TT patients ($p = 0.007$). Meanwhile, for ACPA, they were 85.7% with CC, 87.5% with CT, and only 10% with TT ($p < 0.001$; Tables 5 and 6).

Analysis of genotypes and therapy response

Concerning the relationship of *HLA-DRB1* SE allele and therapy response, *HLA-DRB1* SE0 c had the best therapy response (good response 12.1% and moderate response

Table 4 Relation of HLA-DRB1-SE alleles with clinical and laboratory parameters.

	HLA- DR SE						χ^2	p
	SE0 (n = 66)		SE1 (n = 68)		SE2 (n = 16)			
	No.	%	No.	%	No.	%		
RF								
Negative	30	45.5	9	13.2	3	18.8	18.006*	<0.001*
Positive	36	54.5	59	86.8	13	81.3		
Sig.bet.Grps	$p_1 < 0.001$*, $p_2 = 0.051$, $^{FE}p_3 = 0.691$							
ACPA								
Negative	30	45.5	30	44.1	6	37.5	0.331	0.847
Positive	36	54.5	38	55.9	10	62.5		
Response								
Poor	12	18.2	31	45.6	15	93.8	35.210*	$^{MC}p < 0.001$*
Moderate	46	69.7	35	51.5	1	6.3		
Good	8	12.1	2	2.9	0	0.0		
Sig.bet.Grps	$p_1 = 0.001$*, $p_2 < 0.001$*, $^{MC}p_3 = 0.001$*							

χ^2 chi square test, *FE* Fisher exact, *MC* Monte Carlo, *p* p value for comparing between different categories, p_1 p value for comparing between SE0 and SE1, p_2 p value for comparing between SE0 and SE2, p_3 p value for comparing between SE1 and SE2.

*Statistically significant at $p \leq 0.0028$.

69.7%), while the presence of SE alleles was associated with a dose dependent worsening of therapy response. SE1 patients had (good 2.9%, moderate 51.5%, and poor 45.6%), while SE2 patients had (no good response 0%, moderate 6.3%, and poor 93.8%) ($p < 0.001$; Table 4).

Analysis of the relationship between the disease prognosis using the DAS score difference and *CD28* (*rs1980422*) genotype revealed a statistically significant increase in the frequency of improved patients with the TT genotype (good response 4.2% and moderate response 91.7%) vs. the frequencies of those with CT (good 4.2% and moderate 51%) and CC genotypes (good 16.7% and moderate 36.7%) ($p < 0.001$); meanwhile, there was no significant difference between CT and CC patients (Table 5). Concerning IRF5, the presence of T allele was associated with a significantly better therapy response (good 9.5% and moderate 59.5%) ($p = 0.002$). However, no significant difference in this regard was found between TT, CC, and CT patients (Table 6 and Fig. 1).

Discussion

RA is associated with heterogeneous clinical findings that require different therapeutic strategies. Genetic markers have begun to constitute a promising tool for predicting the response of RA patients to different treatment plans [12, 13]. The aim of this study was to investigate the associations of certain genetic markers (*HLA-DRB1* SE, *CD28 rs1980422*, and *IRF5 rs10488631*) with susceptibility to RA. We also analyzed the associations of these markers with the serological profile of the patient, as well as their associations with disease activity.

Table 5 Relation between CD28 rs1980422 genotype and alleles with different parameters.

	CD28 rs1980422					CD28 rs1980422 (Allele)			
	TT(n = 24)	CT(n = 96)	CC(n = 30)	Test of sig.	p	T(n = 144)	C(n = 156)	Test of sig.	p
RF									
Negative	6 (25.0%)	24 (25.0%)	12 (40.0%)	$\chi^2 = 2.679$	0.262	36 (25.0%)	48 (30.8%)	$\chi^2 = 1.236$	0.266
Positive	18 (75.0%)	72 (75.0%)	18 (60.0%)			108 (75.0%)	108 (69.2%)		
ACPA									
Negative	6 (25.0%)	48 (50.0%)	12 (40.0%)	$\chi^2 = 5.114$	0.078	60 (41.7%)	72 (46.2%)	$\chi^2 = 0.612$	0.434
Positive	18 (75.0%)	48 (50.0%)	18 (60.0%)			84 (58.3%)	84 (53.8%)		
Response									
Poor	1 (4.2%)	43 (44.8%)	14 (46.7%)	$\chi^2 = 23.691$*	$^{MC}<0.001$*	45 (31.3%)	71 (45.5%)	$\chi^2 = 11.517$*	0.003
Moderate	22 (91.7%)	49 (51.0%)	11 (36.7%)			93 (64.6%)	71 (45.5%)		
Good	1 (4.2%)	4 (4.2%)	5 (16.7%)			6 (4.2%)	14 (9.0%)		
Sig.bet.Grps	$^{MC}p_1 < 0.001$*, $^{MC}p_2 < 0.001$*, $p_3 = 0.042$*								

χ^2 chi square test, *H* Kruskal–Wallis test, *U* Mann–Whitney test, *MC* Monte Carlo, *p* p value for comparing between different categories, p_1 p value for comparing between TT and CT, p_2 p value for comparing between TT and CC, p_3 p value for comparing between CT and CC.

*Statistically significant at $p \leq 0.0028$.

Table 6 Relation between IRF5 rs10488631 genotypes and alleles with different parameters.

	IRF5 rs10488631			Test of sig.	p	IRF5 rs10488631		Test of sig.	p
	TT ($n = 60$)	CT ($n = 48$)	CC ($n = 42$)			T ($n = 168$)	C ($n = 132$)		
RF									
Negative	24 (40.0%)	6 (12.5%)	12 (28.6%)	$\chi^2 = 10.013$*	0.007*	60 (35.7%)	24 (18.2%)	$\chi^2 = 11.271$*	0.001*
Positive	36 (60.0%)	42 (87.5%)	30 (71.4%)			108 (64.3%)	108 (81.8%)		
Sig.bet.Grps	$p_1 = 0.0015$*, $p_2 = 234$, $p_3 = 0.057$								
ACPA									
Negative	54 (90.0%)	6 (12.5%)	6 (14.3%)	$\chi^2 = 85.906$*	<0.001*	120 (71.4%)	12 (9.1%)	$\chi^2 = 116.58$*	<0.001*
Positive	6 (10.0%)	42 (87.5%)	36 (85.7%)			48 (28.6%)	120 (90.9%)		
Sig.bet.Grps	$p_1 < 0.001$*, $p_2 < 0.001$*, $p_3 = 0.803$								
Response									
Poor	16 (26.7%)	20 (41.7%)	22 (52.4%)	$\chi^2 = 8.868$	MC0.056	52 (31.0%)	64 (48.5%)	$\chi^2 = 12.199$*	0.002*
Moderate	37 (61.7%)	26 (54.2%)	19 (45.2%)			100 (59.5%)	64 (48.5%)		
Good®	7 (11.7%)	2 (4.2%)	1 (2.4%)			16 (9.5%)	4 (3.0%)		

χ^2 chi square test, *H* Kruskal–Wallis test, *U* Mann–Whitney test, *p* p value for comparing between the studied groups, p_1 p value for comparing between TT and CT, p_2 p value for comparing between TT and CC, p_3 p value for comparing between CT and CC.

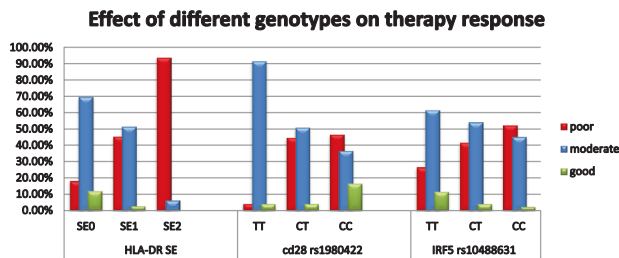*Statistically significant at $p \leq 0.0028$.

Fig. 1 Effect of different genotypes on therapy response. Assessment of therapy response of patients with different genotypes revealed: HLA-DRB1 SE alleles and CD28 rs1980422 polymorphism had a significant negative influence on therapy response; HLA-DRB1 SE2 and SE1 patients had a poor response in (93.8% and 45.6%, respectively), vs. SE0 with a poor response in only 18.2%. While regarding CD28 rs1980422 polymorphism, CC and CT genotype patients had a poor therapy response in (46.7% and 44.8%, respectively), while TT patients had a poor response in only (4.2%). On the other side, IRF5 rs10488631 polymorphism had no effect on therapy response.

In the present study, *HLA-DRB1* SE allele carriers were present at a significantly higher rate among patients (SE1 45.3% and SE2 10.7%) than among controls (SE1 28% and SE2 1.3%). Our findings are in line with several studies providing evidence that the *HLA-DRB1* SE allele is a factor conferring a predisposition for RA [3, 4, 7].

Our results suggest that the *CD28* (*rs1980422*) C risk allele is associated with higher susceptibility to RA. CC and CT were also considered risk genotypes for RA susceptibility (OR = 5.427, CI = 2.586–11.393 and OR = 7.652, CI = 4.263–13.736, respectively), while the TT genotype was considered protective. The association between *CD28 rs1980422* and RA has been established in several studies

[14–16]; however, both Raychaudhuri et al. and Luterek-Puszyńska et al. observed an increased frequency of the C allele in RA patients, although this did not reach significance [5, 17]. This variation in results could be attributed to differences in ethnicity.

Regarding *IRF5* (*rs10488631*), CC and CT were considered risk genotypes (28% of RA patients vs. 4.7% of the control group; and 32% vs. 16.7%, respectively), while the TT genotype was considered to be protective (40% of RA patients vs. 78.6% of healthy controls). The findings of Vernerova et al. agreed with our results, proving that the IRF5 TT genotype is protective against the development of RA [7]. Meanwhile, Wang et al. found no difference in this regard between patients and controls [11].

The serological profile of the patient is considered fundamental to the clinical phenotype of RA, and thus to the choice of treatment [18]. Regarding serological findings, RF seropositivity was associated with both *HLA-DRB1* SE and the *IRF5* risk allele, while no significant association was established with the *CD28* risk allele. However, for ACPA, there was a statistically significant association with only the IRF5 risk allele. The results of Vernerova et al. were in agreement with our results with regard to the genetic associations of RF, but for ACPA they found significant associations with the *HLA-DRB1* SE allele, and *IRF5* and *CD28* risk alleles [7]. Raslan et al. also proved the associations of the *HLA-DRB1* SE allele and RF and ACPA positivity [4].

A genome-wide association study reported that ACPA positivity is associated with only certain members of the SE group of alleles, but not all of them. These findings were illustrated in the form of a model in which the role of MHC-

dependent adaptive immunity is confined mostly to ACPA-positive RA and in which at least two different class II MHC loci may be involved in the pathogenesis of ACPA-positive RA [18]. These findings could explain the discrepancy between our results and other studies.

Identifying biomarkers to choose the correct line of treatment for each patient is considered crucial because of the great variations in treatment options [19]. In this study, we found a better response to methotrexate (MTX) therapy in non-carriers of *HLA-DRB1* SE and those with the *CD28* (*rs1980422*) TT genotype. Patients with the *CD28* (*rs1980422*) TT genotype had the highest rate of improvement in their condition, followed by *HLA-DRB1* SE non-carriers. Despite the fact that these polymorphisms do not directly affect the action of MTX, the presence of these alleles identifies patients with a special clinical profile that might predict disease activity and response to therapy. To our knowledge, these associations have not been previously investigated in any other study. Therefore, it is recommended to conduct further studies on the relationship of these polymorphisms and response to various treatments.

HLA-DRB1 SE, *CD28* (*rs1980422*), and *IRF5* (*rs10488631*) are associated with the risk of developing RA. Moreover, *HLA-DRB1* SE and *CD28* (*rs1980422*) can be used as predictors of the response to MTX therapy.

Subjects and methods

This study was conducted at Zagazig University Hospital, starting at August 2015 till June 2018, and included 150 patients diagnosed with RA who fulfilled the diagnostic criteria for this condition according to the American College of Rheumatology guidelines [20]. In addition, 150 healthy controls with no signs of active inflammatory or infectious diseases were included. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Zagazig University (IRB No.: 1161). Written informed consent was obtained from all subjects.

All patients were diagnosed on the basis of clinical presentation and a general examination with an emphasis on the signs of RA. A detailed examination of the musculoskeletal system was performed for all patients. The disease activity was assessed at the time of sampling using Disease Activity Score 28 (DAS28) [21]. It was based on the number of swollen and tender joints, erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) concentration, and the patient's visual analog scale score. DAS defines the level of RA activity as follows: score ≥5.1 indicates high disease activity; from 5.1 to 3.2 indicates moderate disease activity; between 3.2 and 2.6 indicates low disease activity; and <2.6 indicate clinical remission. All patients received MTX Q6261 (7.5–25 mg per week according to the diseases severity) for

6 months, in addition to non-steroidal anti-inflammatory drugs and low-dose prednisone. Evaluations included DAS28 at the start of treatment and at 6 months. Response to therapy was calculated according to the European League against Rheumatism response criteria, classifying the patients into poor, moderate, and good responders [22].

Exclusion criteria included cases with a history of other autoimmune or inflammatory disorders, pregnancy, and lactation. The demographic and clinical characteristics of all participants are summarized in Table 1.

Sample collection

Venous blood samples were collected in a plain tube and serum was separated and stored in aliquots at −20 °C until the time of assaying. For genotyping, samples were isolated in ethylenediaminetetraacetic acid-containing tubes.

Laboratory assays

ESR was measured by the Westergren method. Serum concentrations of anti-CCP, CRP, and RF were measured using the Cobas E601 analyzer (Roche Diagnostics, Mannheim, Germany) by electrochemiluminescence immunoassay.

Extraction of DNA

Total DNA was extracted from peripheral blood samples using DNA isolation kits (QIAamp DNA Minikit; QIAGEN GmbH, Hilden, Germany), in accordance with the manufacturer's protocol and stored at −20 °C. The concentration of extracted DNA was measured using Qubet fluorometer and assessed by agarose gel electrophoresis. DNA extracted from whole blood samples was genotyped for *IRF5 C/T* (*rs10488631*) and *CD28 C/T* (*rs1980422*) polymorphism by real-time PCR. *HLA-DRB1* allele was determined using Luminex LABScan 100 and LAB Type SSO Class II DRB1 typing kit.

Genotyping for *IRF5* (*rs10488631*) and *CD28* (*rs1980422*)

Genotyping of *IRF5* (*rs10488631*) and *CD28* (*rs1980422*) was performed with a predesigned ready-made primer-probe mixture of one SNP (Custom TaqMan® SNP Genotyping Assays; Thermo Fisher Scientific, USA) and TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, USA), on StepOnePlus™ Real-Time PCR (Thermo Fisher Scientific, USA), following the manufacturer's instructions.

HLA-DRB1 typing

Amplification of the *HLA-DRB1* gene (exon 2) target was performed using LAB Type SSO Class II HLA-DRB1 typing

kit (Hannover, Germany), followed by gel electrophoresis of the PCR product to confirm the presence of the amplicon by visualizing a band at 270 bp. Subsequently, hybridization was performed with complementary DNA probes conjugated to microspheres (beads) labeled with different fluorochromes to identify complementary sequences of the amplified DNA, using the SSO-LAB Type commercial kit (LAB Type SSO Bead Mix; Hannover, Germany) with low to medium resolution, following the manufacturer's protocol. After hybridization, the results were read using the flow cytometry platform LABScan 100 (One Lambda), followed by analysis using the program HLA Fusion version 2.0 (One Lambda). The following alleles were considered as SE alleles: DRB1 *04, *01, and *10. Patients carrying one SE allele were considered SE1 carriers and patients carrying two SE alleles were considered SE2, while patients having no SE alleles were considered SE0 [4].

Statistical analysis

Analysis was carried out using the IBM® SPSS® Statistics software (version 22.0.0.0 IBM Corporation, Armonk, NY, USA. ANOVA (F) test was used to compare three independent groups for normally distributed data. The normally distributed data were analyzed by independent Student's t test, while the Mann–Whitney U test was used for non-parametric values. The Kruskal–Wallis test was used to compare three groups for non-normally distributed data. Genotype frequencies in patients and controls were tested for Hardy–Weinberg equilibrium and difference between observed and expected frequencies were tested for significance. A difference with a p value cut-off was set for 0.0028 as a correction for multiple comparisons.

Compliance with ethical standards

References

1. Imanaka T, Shichikawa K, Inoue K, Shimaoka Y, Takenaka Y, Wakitani S. Increase in age at onset of rheumatoid arthritis in Japan over a 30 year period. Ann Rheum Dis. 1997;56:313–6.
2. Lin L, Chen Y, Xiao Z, Huang S, Yang Z. The association of HLA-DRB1 alleles with rheumatoid arthritis in the Chinese Shantou population: a follow-up study. Biochem Cell Biol. 2007;85:227–38.
3. Oka S, Furukawa H, Shimada K, Hashimoto A, Komiya A, Tsunoda S, et al. Association of HLA-DRB1 genotype with younger age onset and elder age onset rheumatoid arthritis in Japanese populations. Medicine. 2019;98:e18218.
4. Raslan HM, Attia HR, Hamed Ibrahim M, Mahmoud Hassan E, Salama, II, Ismail S, et al. Association of anti-cyclic citrullinated peptide antibodies and rheumatoid factor isotypes with HLA-DRB1 shared epitope alleles in Egyptian rheumatoid arthritis patients. Int J Rheum Dis. 2020;23:647–53.
5. Luterek-Puszyńska K, Malinowski D, Paradowska-Gorycka A, Safranow K, Pawlik A. CD28, CTLA-4 and CCL5 gene polymorphisms in patients with rheumatoid arthritis. Clin Rheumatol. 2017;36:1129–35.
6. Crow MK. Costimulatory molecules and T-cell-B-cell interactions. Rheum Dis Clin North Am. 2004;30:175–91.
7. Vernerova L, Spoutil F, Vlcek M, Krskova K, Penesova A, Meskova M, et al. A combination of CD28 (rs1980422) and IRF5 (rs10488631) polymorphisms is associated with seropositivity in rheumatoid arthritis: a case control study. PLoS ONE. 2016;11:e0153316.
8. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis. 2011;70:2029–36.
9. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet. 2006;38:550–5.
10. García-Bermúdez M, López-Mejías R, Genre F, Castañeda S, Llorca J, González-Juanatey C, et al. Interferon regulatory factor 5 genetic variants are associated with cardiovascular disease in patients with rheumatoid arthritis. Arthritis Res Ther. 2014;16:R146.
11. Wang C, Kokkonen H, Sandling JK, Johansson M, Seddighzadeh M, Padyukov L, et al. Preferential association of interferon regulatory factor 5 gene variants with seronegative rheumatoid arthritis in 2 Swedish case-control studies. J Rheumatol. 2011;38:2130–2.
12. Juge PA, Gazal S, Constantin A, Mariette X, Combe B, Tebib J, et al. Variants of genes implicated in type 1 interferon pathway and B-cell activation modulate the EULAR response to rituximab at 24 weeks in rheumatoid arthritis. RMD Open. 2017;3:e000448.
13. van der Pouw Kraan TC, Wijbrandts CA, van Baarsen LG, Voskuyl AE, Rustenburg F, Baggen JM, et al. Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a sub-population of patients. Ann Rheum Dis. 2007;66:1008–14.
14. Prasad P, Kumar A, Gupta R, Juyal RC, Thelma BK. Caucasian and Asian specific rheumatoid arthritis risk loci reveal limited replication and apparent allelic heterogeneity in north Indians. PLoS ONE. 2012;7:e31584.
15. Kim YO, Kim HJ, Kim SK, Chung JH, Hong SJ. Association of the CD28/CTLA4/ICOS polymorphisms with susceptibility to rheumatoid arthritis. Clin Chem Lab Med. 2010;48:345–53.
16. Ledezma-Lozano IY, Padilla-Martínez JJ, Leyva-Torres SD, Parra-Rojas I, Ramírez-Dueñas MG, Pereira-Suárez AL, et al. Association of CD28 IVS3 +17T/C polymorphism with soluble CD28 in rheumatoid arthritis. Dis Markers. 2011;30:25–9.
17. Raychaudhuri S, Thomson BP, Remmers EF, Eyre S, Hinks A, Guiducci C, et al. Genetic variants at CD28, PRDM1 and CD2/CD58 are associated with rheumatoid arthritis risk. Nat Genet. 2009;41:1313–8.
18. Ding B, Padyukov L, Lundström E, Seielstad M, Plenge RM, Oksenberg JR, et al. Different patterns of associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in the extended

major histocompatibility complex region. Arthritis Rheum. 2009;60:30–8.

19. Viatte S, Barton A. Genetics of rheumatoid arthritis susceptibility, severity, and treatment response. Semin Immunopathol. 2017;39: 395–408.

20. Imboden JB, Hellmann DB, Stone JH. Current diagnosis and treatment. 2013.

21. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995;38:44–8.

22. Salaffi F, Ciapetti A. Clinical disease activity assessments in rheumatoid arthritis. Int J Clin Rheumatol. 2013;8:1758–4272.