CHAPTER 3

FCRL3 promoter 169CC homozygosity is associated with susceptibility to rheumatoid arthritis in Dutch Caucasians

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ABSTRACT

Background: Human leucocyte antigen is the only genetic risk factor for rheumatoid arthritis (RA) that has been consistently observed in different populations. A number of other genes such as PTPN22 and PADI4 showed population-specific association with RA susceptibility. Recently, Fc receptor-like 3 (FCRL3) gene was found to be associated with RA susceptibility in Japanese, but with conflicting results in other populations.

Objective: To investigate the association of FCRL3 polymorphism with RA susceptibility and severity in Dutch Caucasian patients with RA, as well as to perform a meta-analysis to reveal the contribution of this gene to RA susceptibility.

Methods: A total of 931 Dutch RA cases and 570 unrelated Dutch controls were genotyped for four FCRL3 single-nucleotide polymorphisms (SNPs). Genotyping was performed using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry. Association of the FCRL3 SNPs with susceptibility to RA was examined by single-marker, carrier and haplotype analysis.

Results: Carrier analysis of the SNP (rs7528684) revealed the association of CC genotype with a higher risk of developing RA as compared with TT and TC carriers ($p = 0.039$ and OR = 1.31). There was no significant difference in the genotype and allele frequencies of all investigated SNPs between cases and controls. Meta- analysis of all studies comparing 9467 individuals showed that the OR for the CC genotype to develop RA was 1.2 and the p value <0.001.

Conclusion: A promoter polymorphism of FCRL3 (rs7528684) is associated with an increased risk of developing RA in Dutch Caucasians, suggesting that this association is relevant for RA in both Japanese and Caucasian populations.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by chronic inflammation of the synovial joints and hyperplasia and overgrowth of synoviocytes, with consequent destruction. The aetiology and pathogenesis of this disease are not completely understood. Cumulative studies suggest that RA occurs in a genetic background in which multiple common genetic risk factors that may interact are inherited. The most thoroughly examined genes associated with RA are the human leucocyte antigen (HLA) class II gene particularly shared epitope (SE) alleles. However, HLA has been estimated to account for one-third of the genetic component in the disease, indicating that genes outside the HLA region also contribute to the disease [1]. By genome wide scanning and candidate approaches, new candidate susceptibility genes have been identified. Kochi *et al* [2] conducted linkage disequilibrium mapping in a Japanese population using 830 RA cases and 658 controls. An association between susceptibility to RA and a variant of the Fc receptor like 3 gene (FCRL3) was identified. The strongest evidence of association was derived from a polymorphism in the promoter region of FCRL3 (single-nucleotide polymorphism (SNP) rs7528684; 169T/C). The minor C allele was associated with susceptibility to RA, with an odds ratio (OR) of 2.15 (95% CI 1.58 to 2.93), $p<0.001$.

The precise function of FCRL3 is unknown, but its predicted molecular structure suggests that it is a membrane protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif and an immunoreceptor-tyrosine inhibitory motif [3]. Functional analysis showed that presence of the C allele alters a putative nuclear factor kB binding site, and, as a consequence, alters the expression of FCRL3. Moreover, higher expression of FCRL3 was observed in individuals carrying the susceptible allele. Likewise, augmented autoantibody production was associated with the susceptible genotypes, as Kochi *et al* [2] reported a significant association between FCRL3 genotypes and serum rheumatoid factor (RF) level.

In subsequent replication studies in the Japanese population 540 cases and 636 controls, and 748 cases and 934 controls a similar trend was observed regarding allele frequencies and carriership, but with lower p values. These findings did not allow for a conclusion to be

reached on whether the promoter SNP biological effect necessitates a carrier analysis of CC versus TC+TT or C allele analysis [4]. These findings were not replicated in other populations such as American and Spanish Caucasians. Therefore, it is not known whether the association observed in the Japanese is a population-specific effect that is confined to the Japanese or if it is also present in Caucasians [5, 6].

Here, we analysed allele frequencies and carriership in Dutch Caucasian patients with RA and controls. A meta-analysis of the results from Japanese and other populations was performed to gain a better appraisal of the contribution of FCRL3 in the pathogenesis of RA.

METHODS

Subjects

Patients were 975 Dutch Caucasian individuals with RA, all of whom fulfilled the American College of Rheumatology (ACR) classification criteria for RA and have been described elsewhere [7-9]. The main clinical characteristics of the patients were: mean (SD) age at onset 56 (16) years, male:female ratio 2:1, percentage of anticyclic citrullinated peptide antibody (anti-CCP) positivity 59.8%, percentage of shared epitope positivity 67.1% and percentage of erosive disease 56.4%.

Controls were 581 unrelated Dutch Caucasian individuals with no history of RA [10]. Patients and controls were enrolled in the study, with informed written consent obtained according to the Declaration of Helsinki. Commissie Medische Ethiek, the Leiden institutional review board, approved all protocols.

SNPs and genotyping

Two promoter SNPs (rs7528684, rs11264799), one exonic SNP (rs945635) and one intronic SNP (rs10489678) were genotyped using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry, according to the proto- cols recommended by the manufacturer (Sequenom, San Diego, California, USA). SpectroCaller software supplied by the manufacturer was used to automatically call the genotypes. All doubtful calls were checked, and after manually evaluating their spectra, they were accepted, recalled or rejected.

Genotype frequencies for these SNPs were in Hardy–Weinberg equilibrium both in controls and in patients with p values .0.05. The percentage of successful genotyping was .97.1%.

Severity of RA

Radiographs of the hands and feet were scored at baseline, 1 year, 2 years and 4 years using the Sharp–van der Heijde method [11], and were used to assess disease severity and progression. The analysis of these scores was performed only for those patients whose radiographs are available for all time points, in order to avoid bias in the results by missing data.

Anti-CCP

Serum anti-CCP2 was determined by ELISA (Immunoscan RA Mark 2, Euro-diagnostica, Arnhem, The Netherlands) according to the manufacturer's instructions, with a cut-off value of 25 units.

Statistical analysis

A χ^2 test with 2 degrees of freedom was used for association analyses using standard free software package (Epi Info v6, CDC, Atlanta, Georgia, USA). Mann–Whitney U test was used to associate the severity of RA (assessed by radiograph scores) and different genotypes. SPSS V.12.0.1 was used to analyse the data. Haplotypes were predicted using the Chaplin software package v.1.1.3 [12, 13], and linkage disequilibrium (LD) analysis was performed using Haploview [14]. The Easy A 2001 free software package (designed by M Cucherat) was used for the meta-analysis, using the Mantel-Haenszel method for calculating ORs and Breslow and Day method to test for heterogeneity.

RESULTS

Four SNPs located in the FCRL3 gene were genotyped. No significant difference was observed between minor allele frequency and genotypes in patients and controls (**table 1**). Recessive trait comparison of the promoter SNP (rs7528684) (CC versus TT+TC carriers) revealed a significant difference between cases and controls (p = 0.039, OR = 1.31 (95% CI 1.00 to 1.72)) as shown in **table 2**. Comparison of the (TT) genotype carriers with the CC+TC

Table 1 FCRL3—genotype and allele frequencies in patients with Rheumatoid arthritis and controls

SNP reference*	SNP ID†	Genotypes, % (n)			p Value`	Allele frequency (%)		OR (95% CI) 1	p Value
FCRL3_3	rs7528684	TT	TC	CC		T	C		
	Cases (n = 931)	30.9 (288)	46 (428)	23.1 (215)		53.9	46.1		
	Controls (n = 570)	31 (177)	50.4 (287)	18.6 (106)	0.09	56.2	43.8	1.1 (0.94 to 1.28)	0.22
	rs11264799	GG	GA	AA		G	A		
	Cases (n = 941)	52.7 (496)	39.6 (373)	7.7 (72)		72.5	27.5		
	Controls (n = 581)	55.9 (325)	37.4 (217)	6.7 (39)	0.45	74.6	25.4	1.11 (0.94 to 1.32)	0.2
	rs945635	CC	CG	GG		C	G		
	Cases (n = 939)	30.9 (290)	45.3 (425)	23.8 (224)		53.5	46.5		
	Controls (n = 568)	30.5 (173)	50.3 (286)	19.2 (109)	0.065	55.6	44.4	1.09 (0.9 4 to 1.27)	0.257
	rs10489678	GG	GA	AA		G	A		
	Cases (n = 932)	67.5 (629)	28.2 (263)	4.3 (40)		81.6	18.4		
	Controls (n = 571)	67.8 (378)	28.4 (162)	3.8 (22)	0.917	82	18	1.02 (0.84 to 1.25)	0.8

SNP, single-nucleotide polymorphism.
*SNP reference based on Kochi et al.[3]
†Indicates the dbSNP ID as well as the number ofindividuals successfully genotyped.
` x^2 test with 2 degrees offreedom.
1OR and 95% CIs are calculated for the minor allele of each marker.

Table 2 Single-nucleotide polymorphism rs7528684 (169T/C) homozygote minor allele carrier analysis—patients and controls in different studies

Reference	SNP ID*	Genotypes, % (n)		OR (CC vs CT+TT)	P Value†	Allele frequency (%)		OR (95% CI) `	p Value
Current study	rs7528684	CC	CT+TT			C	T		
	Cases (n = 931)	23.1 (215)	76.9 (716)			46.1	53.9		
	Controls (n = 570)	18.6 (106)	81.4 (464)	1.31 (1.00 to 1.72)	0.039	43.8	56.2	1.1 (0.94 to 1.28)	0.22
Kochi et al	rs7528684	CC	CT+TT			C	T		
(initial study)	Cases (n = 824)	19.3 (159)	80.7 (665)			42	58		
	Controls (n = 649)	10 (65)	90 (584)	2.15 (1.56 to 2.97)	0.001	34.5	65.5	1.37 (1.18 to 1.60)	0.000 035
Kochi et al	rs7528684	CC	CT+TT			C	T		
(replication)	Cases (n = 540)	14.3 (77)	85.7 (463)			40.3	59.7		
	Controls (n = 636)	11.8 (75)	88.2 (561)	1.24 (0.87 to 1.78)	0.21	36	63.8	1.19 (1.00 to 1.41)	0.041
Ikari et al[4]	rs7528684	CC	CT+TT			C	T		
	Cases (n = 748)	17.8 (133)	82.2 (615)			43	57		
	Controls (n = 934)	13.8 (129)	86.2 (805)	1.35 (1.03 to 1.77)	0.026	39.1	60.9	1.18 (1.02 to 1.35)	0.022
Hu et al[5]	rs7528684	CC	CT+TT			C	T		
(Set 1)	Cases (n = 467)	18.6 (87)	81.4 (380)			43.4	56.6		
	Controls (n = 473)	20.9 (99)	79.1 (374)	0.87 (0.63 to 1.20)	0.38	44.8	55.2	0.94 (0.78 to 1.14)	0.52
Hu et al	rs7528684	CC	CT+TT			C	T		
(Set 2)	Cases (n = 565)	20.4 (115)	79.6 (450)			45.7	54.3		
	Controls (n = 743)	23 (171)	77 (572)	0.85 (0.65 to 1.12)	0.25	46.8	53.2	0.95 (0.81 to 1.12)	0.55
Martínez et al[6]	rs7528684	CC	CT+TT			C	T		
(Madrid)	Cases (n = 448)	23(102)	77(346)			48	52		
	Controls (489)	21(103)	79(386)	1.10 (0.80 to 1.52)	0.53	45	55	1.15 (0.95 to 1.38)	0.14
Martínez et al	rs7528684	CC	CT+TT			C	T		
(Granada)	Cases (n = 221)	17(38)	83(183)			45	55		
	Controls (n = 229)	18(41)	82(188)	0.95 (0.57 to 1.59)	0.84	43	57	1.09 (0.83 to 1.44)	0.5
Meta-analysis	rs7528684	CC	CT+TT						
of all studies	Cases (n = 4794)	926	3868						
	Controls (n = 4502)	789	3713	1.2 (1.08 to 1.34)	0.001				

SNP, single-nucleotide polymorphism.
*Indicates the dbSNP ID as well as the number ofindividuals successfully genotyped.
†x^2 test with 2 degrees offreedom.
` OR and 95% CIs are calculated for the minor allele of each marker.

carriers (dominant model) showed no significant difference between patients and controls (p = 0.96 and OR = 0.99 (0.79 to 1.25)). No association of (rs7528684) CC genotype was found with SE status, anti-CCP positivity and severity of RA (data not shown). Moreover, we found a significant difference in homozygosity to minor allele between cases and controls in SNP (rs945635; p = 0.034, OR = 1.32 (1.01 to 1.72)). Pairwise LD for the four FCRL3 SNPs was defined using the genotype data from cases and controls separately. The four SNPs were in high LD with each other. Haplotypes were predicted for these four markers. Three haplotypes, each with a frequency >1%, were predicted in both cases and controls, accounting for >96% of all the haplotypes (haplotypes 1 (TGCG), with frequency 53.6% in cases and 55.9% in controls, haplotypes 2 (CAGG), with frequency 27.5% in cases and 25.4% in controls, and haplotypes 3 (CGGA), with frequency 18.5% in cases and 18.4% in controls). Overall, there was no significant association between these FCRL3 haplotypes and disease status (p = 0.2 for haplotype 1, p = 0.2 for haplotype 2 and p = 0.92 for haplotype 3). The two haplotypes carrying the 169C risk allele (haplotypes 2 and 3) were more frequent in patients than in controls, but these differences in frequencies were not significant.

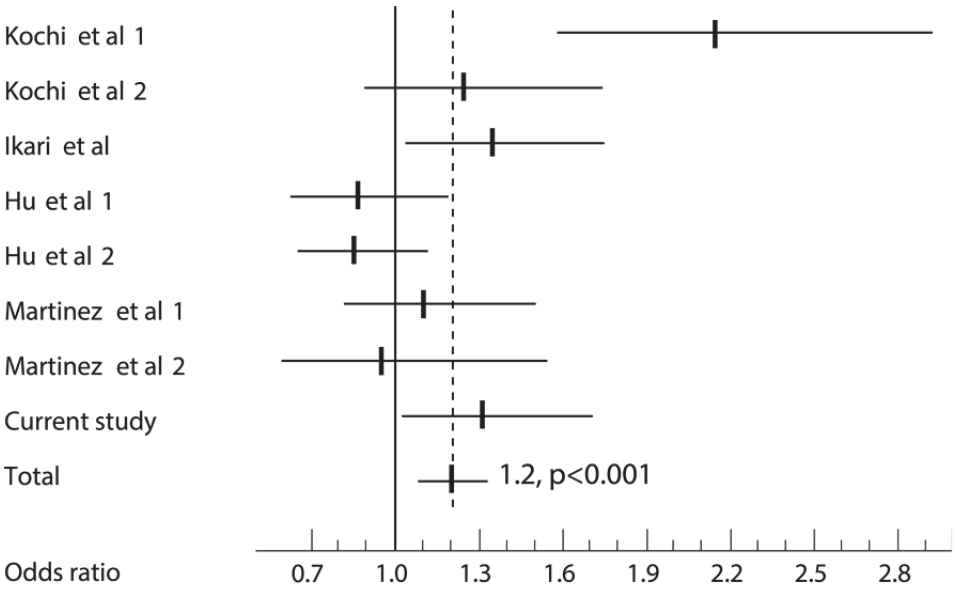

Figure 1 Meta-analysis of different studies on the promoter FCRL3 SNP (rs7528684) in RA.

As no significant differences in allele frequencies were observed between healthy controls in various replication study (Japanese and non-Japanese) compared with the original study of Kochi *et al* [2] (all p values were <0.05), we performed a meta- analysis for the carrier status and its association with RA using all the published studies. A significant difference was observed between CC against CT+TT carriers in 4744 cases and 4723 controls in the combined studied population (p<0.001 and OR = 1.2 (95% CI 1.08 to 1.34); **figure 1**). Meta-analysis of only the Caucasian studies (1600 cases and 1288 controls) similarly showed higher occurrence of CC carriers in patients with RA than in controls, but with a p value of 0.084 (OR = 1.18 (95% CI 0.98 to 1.42)).

DISCUSSION

In the current study, a meta-analysis of all the studies on FCRL3 promoter polymorphism in RA was performed. A total of 9467 patients were studied, and association between the CC carriers and susceptibility to RA was observed, with a p value <0.001 and an OR of 1.2.

Recently, Kochi *et al* [2] reported that an FCRL3 promoter SNP is associated with RA in the Japanese population, and this SNP was shown to alter the binding affinity of nuclear factor kB. Here, we investigated the association of four SNPs within the FCRL3 gene with RA. Our findings partially support the findings in Japanese patients with RA, as we found a significant association between RA and recessive genotype (CC) in the promoter 169T/C SNP. Although the C allele was more frequent in patients with RA than in controls in our study (similar to the findings of Kochi *et al* [2]), the difference was insignificant. Interestingly, a study performed in white North Americans revealed that the minor allele (C) was increased in controls as compared with patients with RA [5]. We also found a significant association between RA and homozygosity to the G allele in the exonic non-coding SNP (rs945635) C/G, but this association is most probably due to the very high LD between this SNP and 169T/C SNP ($r^2 = 0.99$).

Stratification of patients according to anti-CCP and SE status revealed no association between the promoter 169T/C SNP and RA susceptibility in anti-CCP and either SE-positive or SE- negative individuals. Also, no association was found between this promoter SNP and

the severity of RA. We inferred three common FCRL3 haplotypes, but their frequencies were not significantly different between cases and controls.

Our partial replication of the Japanese data, in addition to the failure of other American and Spanish groups to replicate these findings, cannot be explained by the difference in allele frequency between different ethnic groups, as we noticed no significant difference in allele frequency in controls between all the studied groups from different ethnic backgrounds. Likewise, we do not consider it likely that the difference in disease severity between different studied groups, in case this variant of FCRL3 is associated with severity, explains the variation found between different studied populations, because, in our study, no association between disease severity and the allele or genotype frequency in the promoter 169T/C SNP was observed; however, we could find an association between the recessive trait (CC) and RA.

A possible explanation for the difference in findings between different studies is related to the sizes of the studied groups. Until now, a total of 9467 individuals have been studied (in all the studies), and the meta-analysis indicates a significant difference between carriers of the recessive trait genotype in patients and controls ($p<0.001$ and OR = 1.2). Additionally, even though the allele frequency of the promoter SNP was not significantly different between all populations, there might be other hidden factors influencing the effect of this SNP, which are specific for a particular ethnic group in the various studied populations. In our study, we analysed FCRL3 polymorphism in RA as a recessive trait (CC vs TC+TT) and we partially replicated the Japanese findings. This study is the first positive replication study in Caucasians, but, given the heterogeneity of results from different studies, additional well-powered studies in Caucasians are necessary to clear the remaining doubt regarding the validity of the association of FCRL3 with RA susceptibility. Taking into consideration that all the European studies on FCRL3 promoter polymorphism in RA, including ours, showed no differences in allele and genotype frequencies between patients and controls, future studies might consider the possible involvement of FCRL3 promoter polymorphism in RA in a recessive trait fashion rather than by allele frequency comparison.

CONCLUSIONS

We conclude that the FCRL3 promoter SNP is associated with susceptibility to RA in individuals who carry the recessive genotype CC in Dutch Caucasians. These findings suggest that the association of FCRL3 with RA susceptibility might not be specific to the Japanese population, and that FCRL3 is possibly involved in the pathogenesis of RA in a recessive trait fashion. Further well-powered studies are needed to clarify this association in various ethnic groups.

Note added in proof: Shortly after this manuscript was accepted for publication, Newman *et al* [15] published a study that concluded that RA association with the FCRL32169C polymorphism is restricted to PTPN22 1858T-homozygous individuals in a Canadian population [15]. Accordingly, we performed a similar analysis in our cohort and found no statistically significant difference in FCRL3 169C variants between patients carrying and those lacking the PTPN22 1858T risk allele (data not shown). Hence, we conclude that, in our cohort, there is no interaction between FCRL3 and PTPN22 risk alleles in predisposing to RA.

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