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ANALYSIS OF THE FC-RECEPTOR LIKE-3 (*FCRL3*) LOCUS IN CAUCASIANS WITH AUTOIMMUNE DISORDERS SUGGESTS A COMPLEX PATTERN OF DISEASE ASSOCIATION

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Abstract

Context: A four marker haplotype in the 5' region of the Fc receptor-like 3 gene (markers *FCRL3_3* to *FCRL3_6*) has recently been identified as contributing to rheumatoid arthritis susceptibility in the Japanese population. The promoter *FCRL3_3*C* allele also showed significant association with autoimmune thyroid disease and systemic lupus erythematosus. These findings raise the possibility that this locus may influence autoimmune disease susceptibility across many populations.

Patients and Design: We analysed the same four 5' *FCRL3* SNP markers, together with three additional exonic SNPs in the *FCRL3* gene, in cohorts of white Caucasians with Graves' disease (n=625), type 1 diabetes (n=279), autoimmune Addison's disease (n=200) and rheumatoid arthritis (n=769). Healthy controls from the UK (n=490) and New Zealand (n=593) were used.

Results: Six of the seven *FCRL3* markers showed association with autoimmune Addison's disease (p=0.005 to 0.0001), with maximum evidence at the *FCRL3_3*T* allele ($p_{[corrected]}$=0.0008; odds ratio 1.61; 5-95% confidence intervals 1.26 to 2.05). The most common seven marker *FCRL3* haplotype (TGGGAAA) was also found to be significantly associated with autoimmune Addison's disease $p_{[corrected]}=1.1x10^{-4}$; odds ratio 1.71; 5-95% confidence intervals 1.33-2.18). There was nominal evidence for allelic association at the marker *FCRL3_8* in Graves' disease (OR 1.50; 5-95% CI 1.06-2.13) and at *FCRL3_9* with rheumatoid arthritis (OR 1.25; 5-95% CI 1.01-1.54).

Conclusions: The *FCRL3* haplotype that is associated with autoimmune Addison's disease in Caucasians appears to be protective for autoimmune diseases in the Japanese population, demonstrating that this haplotype is unlikely to contain a single primary etiological allele for autoimmunity. Our observations suggest that the susceptibility to autoimmunity at the *FCRL3* locus is more complex than initially thought, and may extend either side of the currently associated region, to include the adjacent *FCRL2* gene.

Introduction

Autoimmune endocrinopathies, together with immune-mediated rheumatological disorders contribute the major burden of disease caused by autoimmunity in the population, with around 5% of women in developed societies being affected by such conditions. All the common autoimmune conditions have a complex genetic basis, with autoimmune endocrinopathies and rheumatoid arthritis (RA) commonly occurring in the same individual and clustering within families [1]. Graves' disease (GD), RA, and type 1 diabetes (T1D) are frequent disorders (prevalence 0.4-1%), and each has a λs (ratio of risk to sibling vs unrelated background population) of between 7 and 15, suggesting a significant genetic component to susceptibility. In contrast, autoimmune Addison's disease (AAD) is a much rarer condition with a prevalence of about 1 per 10,000 people in the UK [2] but a risk to first-degree relatives of about 2%, suggesting a more marked genetic influence on disease susceptibility. Whole-genome linkage studies have shown clustering of susceptibility loci for many of these different disorders and several loci consistently appear to contribute to multiple forms of autoimmunity across diverse populations. Examples of such loci include the major histocompatibility complex (*MHC*) [3,4], the cytotoxic T lymphocyte antigen-4 gene (*CTLA4*) [5-10] and the protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*) [11-13]. A second class of autoimmunity susceptibility locus is represented by the disease-specific loci, such as the insulin gene (*INS*, *IDDM2*) [14], or the thyrotropin receptor [15,16] whose contribution is unique to the relevant target tissue or specific antigen of the autoimmune response. A final class of loci are those where replicable disease associations are found in some populations studied, but for which there appears to be little or no contribution to disease in well-powered studies of other populations. For example the *PADI4* gene is associated with RA in the Japanese population [17-19] but with a weak effect, only demonstrable by meta-analysis, in European populations [20-22]. Thus, it remains imperative to study putative autoimmune disease susceptibility alleles in different autoimmune conditions and also in several populations of varied ethnic background. The latter approach,

sometimes termed “transracial mapping” can give important clues to the identity of aetiologically important alleles [23].

One recently identified novel susceptibility gene in rheumatoid arthritis is the Fc receptor-like 3 gene (*FCRL3*) [24], located at 1q21, a region implicated in susceptibility to several autoimmune diseases in whole-genome linkage studies [25-27]. Kochi et al. identified a four single nucleotide polymorphism (SNP) haplotype (*FCRL3_3 to FCRL3_6*) as being associated with RA in two separate cohorts of Japanese patients. The greatest association with RA ($p=8.5x10^{-7}$; odds ratio 2.15) was found in individuals who were homozygote carriers of a particular promoter allele, *FCRL3_3*C*, at position -169 relative to the *FCRL3* transcription start site. This allele (*FCRL3_3*C*) was also found to be associated in Japanese cohorts with autoimmune thyroid disease ($p=1.7x10^{-5}$; odds ratio 1.74) and systemic lupus erythematosus (p=0.0017; odds ratio 1.49). This same allele was also found to produce higher promoter activity in a reporter gene assay and to be more avidly bound by NFκB in gel-shift studies, suggesting a direct functional role [24]. FCRL3 is an orphan cell-surface receptor with homology to the Fc immunoreceptors, and is expressed predominantly in B-lymphocytes in lymph node germinal centres. In this study we have analysed the same 4 marker *FCRL3* SNP haplotype and 3 additional exonic SNP markers in four cohorts of white Caucasians with autoimmune disorders including GD, AAD, RA and T1D.

Materials and Methods

Subjects

The GD (n=625) and AAD (n=105) probands were recruited through endocrine and combined physician-ophthalmologist thyroid associated ophthalmopathy clinics at the Newcastle upon Tyne Hospitals Trust and surrounding district hospitals. A further cohort of 95 AAD probands were recruited via the UK Addison’s disease self-help group. The diagnostic criteria of these cohorts

have been published previously [28,29]. Of the GD probands 78% were female, 38% had significant thyroid associated opthalmopathy (NOSPECS class 3 or worse) and 55% were smokers. The UK GD subjects all had parents born in the North-East of England. Isolated AAD accounted for 36% of the AAD cohort, the other 64% had at least one other associated autoimmune disease (hypothyroidism, 77; Graves' disease, 24; primary gonadal failure, 23; type 1 diabetes, 12; pernicious anaemia, 13; vitiligo, 6; celiac disease, 6; rheumatoid arthritis, 4; alopecia, 3; haemolytic anaemia, 2; and autoimmune hepatitis, 1). The AAD cohort included 151 females (75.5%) and 49 males (24.5%). The mean age of onset was 40 years old. Four of the 200 AAD probands also had an affected first degree relative with AAD (two siblings, two offspring). None of the AAD subjects had autoimmune hypoparathyroidism or candidiasis (subjects with type 1 polyendocrinopathy were excluded from the cohort) [28]. UK controls (n=490; 66.3% females, 33.7% males) also recruited from the local population had no clinical features or family history of autoimmune disease. The RA (n=769) cases were recruited from rheumatology clinics throughout New Zealand and details of their clinical characterisation, and of the NZ control cohort (n=563) have previously been published [13]. The T1D cohort comprised 279 subjects with hyperglycaemia and ketosis who were commenced on insulin therapy at diagnosis, recruited from endocrinology clinics throughout New Zealand. They had an average age of onset of 12.5 years.

SNP genotyping

The SNPs within the 4-marker haplotype described by Kochi et al (2005) [24] were genotyped either by PCR and restriction enzyme digest (RFLP) (UK cohort SNPs *FCRL3_3, 3_4*; NZ cohort *FCRL3_3* to *3_6*) or primer extension-MALDI-TOF assay (Sequenom, Inc., San Diego, CA) (UK cohort SNPs *FCRL3_5, 3_6*). Furthermore, we examined three additional exonic *FCRL3* SNPs, 2 of which are non-synonymous cSNPs. The additional markers were located as follows: rs7522061 -exon 3 cSNP (D28N); rs2282284 (*FCRL3_8*) exon 14 cSNP (N721S); rs2282283

(*FCRL3_9*) -3'UTR (Figure 1) and were genotyped by PCR/RFLP (UK cohort SNP D28N; NZ cohort D28N, *FCRL3_8 & 3_9*) or primer extension-MALDI-TOF assay (UK cohort SNPs *FCRL3_8, 3_9*). Details of the assay oligonucleotide sequences and conditions are available from the authors *(shown in supplementary table 1*).

Statistical analysis

The case-control association studies were analysed using χ^2 tests on 2x2 and 2x3 contingency tables for allele and genotype frequencies, respectively. Haplotype frequencies were estimated, and linkage disequilibrium (r^2) measures were calculated using the SHEsis package [30]. Odds ratios and confidence intervals were calculated using Woolf's method. No significant deviation from Hardy Weinberg equilibrium was observed for any of the SNPs in this study (all $p>0.05$). The overall genotype call rate was 98.2% (range 91.6-100%), and the accuracy was >99% according to duplicate genotyping of 7-10% of samples. We estimate that our studies of GD and RA had more than 80% power to detect an effect ($\alpha=0.001$) of the same magnitude (allelic odds ratio of 1.4) as that found in the Japanese GD cohorts [24], using our control allele frequencies and a binomial model. The power of our studies of AAD and T1D were >80% and >90%, respectively, assuming an allelic odds ratio of 1.4 ($\alpha=0.05$). P values were Bonferroni corrected for multiple tests assuming that the 7 SNP markers carried information for 4 independent linkage groups, and that each of the 4 disease states were independent.

Results

We found significant linkage disequilibrium between markers at the 5' end of *FCRL3,* but lesser association between the other 3' alleles, with pairwise r^2 values based on the UK control population as follows: *FCRL3_3* – [0.412]– *FCRL3_4* – [0.410] –*FCRL3_5* – [0.987] – *FCRL3_6* – [0.915] – N28D – [0.088] – *FCRL3_8* – [0.024] – *FCRL3_9* (Figure 1). The corresponding

figures for the NZ control population are: *FCRL3_3* – [0.396]– *FCRL3_4* – [0.420] –*FCRL3_5* – [0.834] – *FCRL3_6* – [0.749] – N28D – [0.068] – *FCRL3_8* – [0.011] – *FCRL3_9*. In contrast to the finding of strong association with disease of the *FCRL3_3* to *FCRL3_6* SNPs in Japanese RA and GD subjects, we could find no evidence to support disease association with alleles at any of these markers in UK whites with GD or in NZ whites with RA or T1D (Table 1). Markers in the 3' end of the gene showed nominal evidence for association at *FCRL3_8* in Graves' disease (A allele, OR 1.50; 5-95% confidence intervals 1.06-2.13) and at *FCRL3_9* in RA (C allele, OR 1.25; 5-95% confidence intervals 1.01-1.54), however, these findings were not robust to correction for multiple statistical testing (Table 1).

In contrast, there was substantial allelic association at 6 of the 7 *FCRL3* markers studied in the cohort of UK subjects with autoimmune Addison's disease, with maximum evidence at *FCRL3_3*. The T allele of this marker *(FCRL3_3*T*) was present in 254 of 400 (63.5%) AAD chromosomes compared to 467 of 898 (52.0%) control chromosomes ($p_{[corrected]}$=0.0008; OR 1.61; 5-95% confidence intervals 1.26 to 2.05) (Table 1). The most common seven marker haplotype containing the *FCRL3_3*T* allele [TGGGAAA] was also significantly associated with AAD, p=1.8 $x10^{-5}$, $p_{[corrected]}$=1.1$x10^{-4}$ (odds ratio 1.71; 5-95% confidence intervals 1.33-2.18), with a p value of 0.001 using a global test of the 6 common haplotypes found in the AAD patients (Table 2). There were no significant haplotype associations with disease in either of the GD, RA or T1D cohorts (Table 2). There was no significant heterogeneity in the allelic associations in the various cohorts when divided by diagnostic subgroups *(supplementary table 2)*.

Discussion

The association of multiple autoimmune disorders in the Japanese population with alleles of *FCRL3* by Kochi and colleagues [24] suggested that this locus may affect susceptibility to different autoimmune diseases across many populations, in a similar way to the action of alleles

at *CTLA4* [5-10]. The association of the *FCRL3_3*C* allele with susceptibility to autoimmune conditions found in this initial study has recently been replicated in a further cohort of Japanese subjects with rheumatoid arthritis (n=752) [31]. Furthermore, a study of a white UK population showed a weak effect in Graves' disease, with *FCRL3* markers having nominal association (*FCRL3_3*C,* p=0.024, OR 1.17) [32]. However, similar investigation of several additional large cohorts of Caucasian subjects with T1D and RA have failed to replicate the *FCRL3_3* association [33-36]. In contrast to these studies, our investigation found that the *FCRL3_3*C* allele was associated with protection from autoimmune Addison's disease; an observation that largely refutes the hypothesis that this allele is the disease-causing polymorphism for autoimmunity at this locus, at least not in Caucasians. In addition, our extended genotyping points to the exon 3 cSNP (D28N) as also being tightly associated with *FCRL3_3* ($r^2 > 0.9$; Figure 1) and this marker certainly warrants examination in Japanese and other autoimmune disease cohorts. While our analyses of rheumatoid arthritis and Graves' disease were well-powered to detect an effect of similar magnitude to that found in the Japanese cohorts, only marginal evidence for association was found in these conditions; this being found at the 3' *FCRL3* SNPs (designated *FCRL3_9* and *FCRL3_8*, respectively), which are not in the haplotype block containing the promoter and exon 3 markers (Figure 1).

The contradiction of the apparent protection conferred in the Addison's disease cohort by the Japanese autoimmune susceptibility haplotype could be explained in three ways. Firstly, the association reported here in the AAD cohort could be a chance finding. It is important that other AAD cohorts are genotyped for the *FCRL3* variants analysed here. However, genotype data at other loci do not support population stratification or mismatching between the AAD cohort and UK controls as an explanation for this result [37,38; *supplementary table 3*]. Secondly, the disparate *FCRL3* genetic association could reflect genuine differences in *FCRL3*-mediated autoimmune disease aetiology between the Japanese and Caucasian populations. In addition, it is

possible that different immunopathological mechanisms underlie the disease process in AAD compared to RA, GD and the other more common autoimmune diseases. With this regard, the lower promoter activity of the FCRL3_3*T allele that would be predicted based on the existing functional analysis at this locus [24], could have a distinct pathogenic role in AAD, but still be protective for other forms of autoimmunity. Lastly, the susceptibility allele at *FCRL3* may lie elsewhere within the block of linkage disequilibrium that contains the 5' region of the *FCRL3* gene, or within the adjacent *FCRL2* transcript (figure 1). The 5' region of *FCRL3* is in an LD block which extends across an intragenic region to the 3' end of the *FCRL2* gene, including several coding *FCRL2* exons. In addition, the 3' region of *FCRL3* (centromeric to exon 5 and encoding the critical cytoplasmic tail of the receptor) is sparsely covered by existing markers and appears to contain a substantial interval with weak or no LD between markers [39]. Furthermore, there may be additional subtle differences in LD structure between Japanese and Caucasians at this locus that will not be evident until a denser marker-map is genotyped. Evidence for association of *FCRL3* promoter alleles with autoimmune disease is currently consistent in Japanese populations but equivocal or absent in most Caucasian populations (32-36). To further clarify a possible role for this locus in autoimmunity in Caucasians, our findings suggest that more detailed genetic analysis of an extended haplotype block containing both *FCRL3* and *FCRL2* is warranted.

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Legend for figure 1. Linkage disequilibrium r^2 measures for *FCRL3* in the UK control population. In the schematic diagram of the *FCRL3* gene, exons are depicted by black boxes and non-coding regions as white boxes. The position of the seven SNPs (*FCRL3_3,-4,-5,-6*, N28D, -*8,-9*) spanning 22.435 kb of the *FCRL3* gene region are shown.

Table 1. Genotype and allele data for each of the seven *FCRL3* single nucleotide polymorphisms (SNPs) in each population studied, together with case control analysis of the alleles and genotypes in each disease group in comparison to the relevant controls.

Marker	Population	Allele (1/2)	Genotype number (%)			Allele number (%)		p values*		
			1 1	1 2	2 2	1	2	Genotypes	Alleles	Alleles (p corrected)
FCRL3_3 (promoter rs7528684	UK controls	C/T	97 (21.6)	237 (52.8)	115 (25.6)	431 (48.0)	467 (52.0)			
	UK AAD	C/T	28 (14.0)	90 (45.0)	82 (41.0)	146 (36.5)	254 (63.5)	0.0002	0.0001	0.0008
	UK GD	C/T	132 (21.7)	304 (50.0)	172 (28.3)	568 (46.7)	648 (53.3)	0.58	0.56	-
	NZ controls	C/T	125 (22.6)	275 (49.7)	153 (27.7)	525 (47.5)	581 (52.5)			
	NZ RA	C/T	168 (22.1)	386 (50.7)	207 (27.2)	722 (47.4)	800 (52.6)	0.94	0.99	-
	NZ T1D	C/T	65 (24.6)	117 (44.3)	82 (31.1)	247 (46.8)	281 (53.2)	0.35	0.79	-
FCRL3_4 (promoter) rs11264799	UK controls	A/G	48 (10.2)	194 (41.5)	226 (48.3)	290 (31.0)	646 (69.0)			
	UK AAD	A/G	10 (5.0)	66 (33.0)	124 (62.0)	86 (21.5)	314 (78.5)	0.002	0.0004	0.0032
	UK GD	A/G	54 (8.8)	252 (41.0)	308 (50.2)	360 (29.3)	868 (70.7)	0.67	0.40	-
	NZ controls	A/G	44 (7.8)	223 (39.7)	295 (52.5)	311 (27.7)	813 (72.3)			
	NZ RA	A/G	51 (6.6)	277 (36.0)	441 (57.3)	379 (24.6)	1159 (75.4)	0.20	0.08	-
	NZ T1D	A/G	21 (7.8)	100 (37.2)	148 (55.0)	142 (26.4)	396 (73.6)	0.77	0.58	-
FCRL3_5 (5'UTR) rs945635	UK controls	C/G	103 (22.0)	245 (52.2)	121 (25.8)	451 (48.1)	487 (51.9)			
	UK AAD	C/G	29 (14.5)	91 (45.5)	80 (40.0)	149 (37.3)	251 (62.7)	0.0007	0.0003	0.0024
	UK GD	C/G	149 (24.2)	300 (48.7)	167 (27.1)	598 (48.5)	634 (51.5)	0.50	0.83	-
	NZ controls	C/G	120 (21.4)	287 (51.2)	154 (27.5)	527 (47.0)	595 (53.0)			
	NZ RA	C/G	161 (21.1)	383 (50.1)	220 (28.8)	705 (46.1)	823 (53.9)	0.86	0.67	-
	NZ T1D	C/G	55 (20.4)	130 (48.3)	84 (31.2)	240 (44.6)	298 (55.4)	0.53	0.37	-
FCRL3_6 (Intron 2) rs3761959	UK controls	A/G	100 (22.1)	236 (52.1)	117 (25.8)	436 (48.1)	470 (51.9)			
	UK AAD	A/G	29 (14.5)	91 (45.5)	80 (40.0)	149 (37.3)	251 (62.7)	0.0007	0.0003	0.0024
	UK GD	A/G	150 (24.7)	294 (48.3)	164 (27.0)	594 (48.8)	622 (51.2)	0.45	0.74	-
	NZ controls	A/G	136 (24.2)	277 (49.4)	148 (26.4)	549 (48.9)	573 (51.1)			
	NZ RA	A/G	155 (20.3)	386 (50.7)	221 (29.0)	696 (45.7)	828 (54.3)	0.21	0.10	-
	NZ T1D	A/G	58 (21.5)	135 (50.0)	77 (28.5)	251 (46.5)	289 (53.5)	0.63	0.35	-
N28D (Exon 3) rs7522061	UK controls	G/A	107 (22.9)	249 (53.3)	111 (23.8)	463 (49.6)	471 (50.4)			
	UK AAD	G/A	31 (15.5)	93 (46.5)	76 (38.0)	155 (38.8)	245 (61.2)	0.0005	0.0003	0.0024
	UK GD	G/A	159 (26.1)	301 (49.3)	150 (24.6)	619 (50.7)	601 (49.3)	0.38	0.59	-
	NZ controls	G/A	117 (20.9)	290 (51.8)	153 (27.3)	524 (46.8)	596 (53.2)			
	NZ RA	G/A	155 (20.4)	381 (50.1)	224 (29.5)	691 (45.5)	829 (54.5)	0.69	0.50	-
	NZ T1D	G/A	55 (21.0)	126 (48.1)	81 (30.9)	236 (45.0)	288 (55.0)	0.52	0.51	-
FCRL3_8	UK controls	G/A	3 (0.6)	73 (14.9)	414 (84.5)	79 (8.1)	901 (91.9)			

(Exon 14) rs2282284	UK AAD	G/A	0 (0)	15 (7.7)	180 (92.3)	15 (3.8)	375 (96.2)	0.02	0.005	0.04
	UK GD	G/A	1 (0.2)	66 (10.7)	550 (89.1)	68 (5.5)	1166 (94.5)	0.05	0.02	0.16
	NZ controls	G/A	3 (0.5)	57 (10.1)	503 (89.3)	63 (5.6)	1063 (94.4)			
	NZ RA	G/A	1 (0.1)	77 (10.1)	688 (89.8)	79 (5.2)	1453 (94.8)	0.42	0.62	-
	NZ T1D	G/A	0 (0)	24 (8.6)	255 (91.4)	24 (4.3)	534 (95.7)	0.36	0.26	-
FCRL3_9 (3'UTR) rs2282283	UK controls	C/A	27 (5.5)	158 (32.3)	304 (62.2)	212 (21.7)	766 (78.3)			
	UK AAD	C/A	9 (4.6)	53 (26.9)	135 (68.5)	71 (18.0)	323 (82.0)	0.29	0.13	-
	UK GD	C/A	31 (5.0)	214 (34.2)	380 (60.8)	276 (22.1)	974 (77.9)	0.76	0.82	-
	NZ controls	C/A	20 (3.6)	139 (24.8)	402 (71.7)	179 (16.0)	943 (84.0)			
	NZ RA	C/A	34 (4.4)	226 (29.5)	507 (66.1)	294 (19.2)	1240 (80.8)	0.10	0.03	0.24
	NZ T1D	C/A	9 (3.4)	65 (24.8)	188 (71.8)	83 (15.8)	441 (84.2)	1.00	0.95	-

AAD, autoimmune Addison's disease; GD, Graves' disease; NZ, New Zealand; RA, rheumatoid arthritis; T1D, type 1 diabetes
* p values were calculated by comparing the allele or genotype numbers for each disease cohort with those from the relevant controls. P values were Bonferroni corrected (p corrected) for multiple testing as described in *methods*.

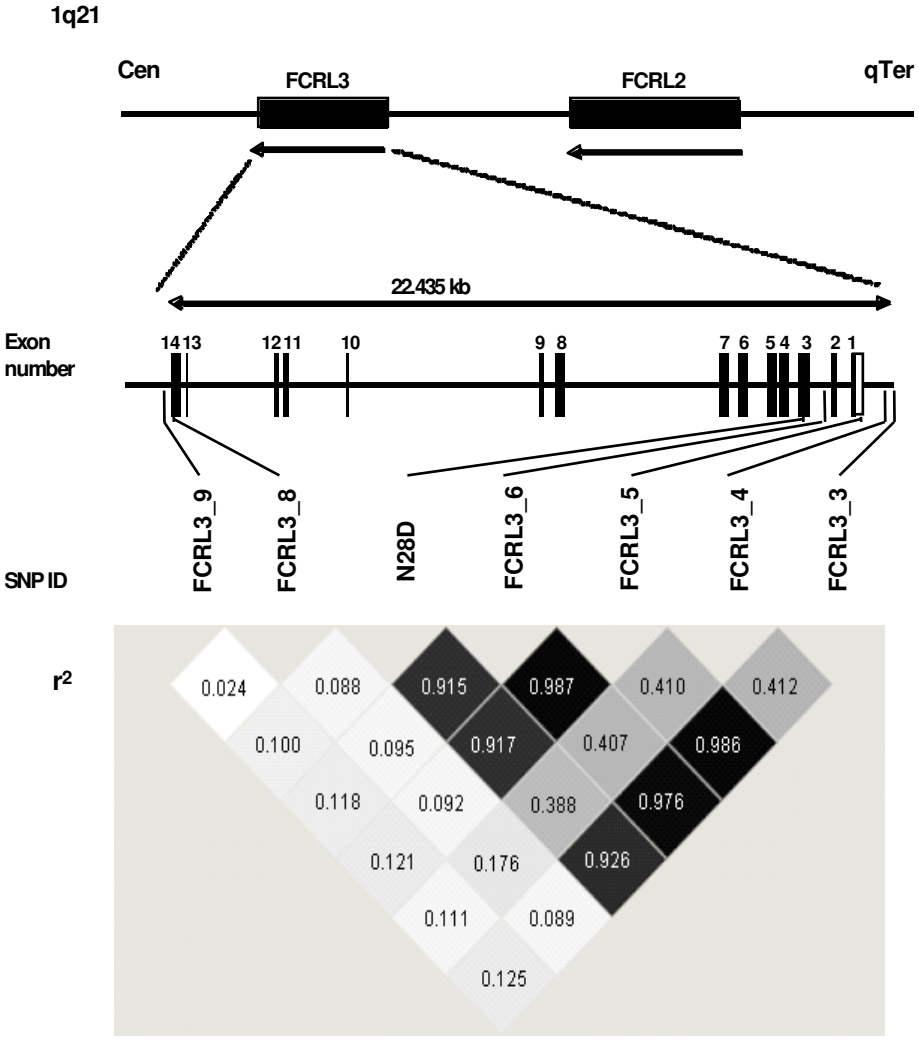
Table 2. Haplotype structures and frequencies of *FCRL3* in the different populations with a frequency of >1%. Significance levels (p) for the UK Addison's disease cohort compared to UK controls are also shown. None of the other populations showed any association with the *FCRL3* haplotypes.

Haplotype	**Sequence (*FCRL3_3-4-5-6-N28D-8-9*)**	**Frequency**						
		UK controls	**UK AAD**	**p value * (AAD v controls)**	**UK GD†**	**NZ controls**	**NZ RA†**	**NZ T1D†**
1	TGGGAAA	0.46	0.59	1.8×10^{-5}	0.45	0.44	0.43	0.45
2	TGGGAAC	0.04	0.02	0.21	0.03	0.03	0.04	0.03
3	TGGGGAA	0.02	0.02	0.93	0.03	0.02	0.02	0.02
4	CACAGAA	0.20	0.17	0.17	0.23	0.21	0.18	0.21
5	CACAGGA	0.08	0.04	0.01	0.05	0.05	0.04	0.03
6	CGCAGAC	0.19	0.15	0.15	0.18	0.12	0.15	0.11
7	CGCAGAA	-	-	-	-	0.05	0.04	0.04
		Global p value		0.001				

*The p value for the association of the TGGGAAA haplotype in AAD is 1.1×10^{-4}, when corrected for the 6 haplotypes observed.

† no significant association of any of the *FCRL3* haplotypes in these patient cohorts in comparison to the relevant controls.

AAD, autoimmune Addison's disease; GD, Graves' disease; NZ, New Zealand; RA, rheumatoid arthritis; T1D, type 1 diabetes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