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Association analysis of *IL1A* and *IL1B* variants in alopecia areata

RACHID TAZI-AHNINI*†‡, ANDREW J. G. McDONAGH‡, ANGELA COX†, ANDREW G. MESSENGER‡, JAMES E. R. BRITTON‡, SIMON J. WARD†, CLAES O. BÅVIK†, GORDON W. DUFF† & MICHAEL J. CORK†‡

†Biomedical Genetics Project, Division of Genomic Medicine and ‡Department of Dermatology, University of Sheffield, Royal Hallamshire Hospital, Sheffield S10 2JF, U.K.

Alopecia areata is an inflammatory hair loss disease with a major genetic component. The disease is characterized by focal inflammatory lesions with perifollicular T-cell infiltrates, reflecting the role of local cytokine production in the development of patchy hair loss. IL-1α and IL-1β are important inhibitors of hair growth *in vitro*. Their effect is opposed by the interleukin-1 receptor antagonist, IL-1ra. Genes of the IL-1 cluster are candidate genes in the pathogenesis of alopecia areata. To investigate the role of the IL-1 system in alopecia areata we examined three biallelic polymorphisms within the IL-1 gene cluster (*IL1A* + 4845, *IL1B* + 3954 and *IL1B*–511) in 165 patients and a large number of matched controls ($n = 1150$). There was no significant association of *IL1B*–511 or *IL1B* + 3954 genotypes with the overall dataset, or with disease severity or age at onset, in contrast with a previous report. The results suggested the possibility of an association with *IL1A* + 4845 in the overall dataset [OR 1.39 (95% CI 1.00, 1.93)] although this was not statistically significant. This was due mainly to the contribution from mild cases of alopecia areata [OR 1.48 (0.96, 2.29)], suggesting that IL-1α may have a particular role in the pathogenesis of this subgroup.

Keywords: alopecia areata, HLA, *IL1A*, *IL1B*, interleukin-1, polymorphism.

Introduction

Alopecia areata is characterized by patchy hair loss with perifollicular and intrafollicular T-cell infiltration (Kalish *et al*., 1992; McDonagh & Messenger, 1996). The association of alopecia areata with autoimmunity, including thyroid disorders, pernicious anaemia and vitiligo, is well established (Muller & Winkelmann, 1963; Cunliffe *et al*., 1968) and alopecia areata itself is conventionally regarded as an autoimmune disease.

There are no reliable population-based data on the prevalence of alopecia areata in the UK but the lifetime risk of disease is thought to be about 1.7% in the USA with similar incidence in males and females (Safavi *et al*., 1995). Up to 40% of individuals with alopecia areata have a positive family history of the disease (Anderson, 1950; van der Steen *et al*., 1992) and twin studies (Cole & Herzlinger, 1984; Scerri & Pace, 1992) have confirmed that there is a genetic component to the disorder. Several investigators have shown an association between alopecia areata and particular HLA class I alleles including *A28*, *B12*, *B13*, *B18*, *B27* and *Cw3* (Kianto *et al*., 1977; Hacham-Zadeh *et al*., 1981; Orecchia *et al*., 1987; Price & Colombe, 1996); but HLA Class II associations including *DR4*, *DR11*, *DPw4*, *DQw3*, *DQw7* and *DQw8* are considered more important (Frentz *et al*., 1986; Odum *et al*., 1990; Duvic *et al*., 1991). A high relative risk of disease for HLA DR5 ($RR = 3.14$, $P < 0.01$) was found in patients with severe disease and early age at onset (Price & Colombe, 1996) and the importance of HLA genes has been confirmed in the only family study reported to date (de Andrade *et al*., 1999). However, the HLA contribution alone cannot explain the entire genetic basis of alopecia areata, and we have found evidence to suggest the presence of another alopecia areata locus on chromosome 21 (Tazi-Ahnini *et al*., 2000). Interaction between *HLA* and other loci is probably required to produce the disease phenotype, as shown for systemic lupus erythematosus (Tjernström *et al*., 1999).

Interleukin-1 is a highly pro-inflammatory cytokine that promotes recruitment of T-lymphocytes, neutrophils and macrophages to inflamed tissues (Dinarello, 1996). IL-1α, IL-1β and TNF-α are also known to have an inhibitory effect on hair growth *in vitro* (Harmon & Nevins, 1993; Philpott *et al*., 1996). Inhibitory doses of

*Correspondence. E-mail: r.taziahnini@sheffield.ac.uk

these three cytokines have similar effects on the morphology of cultured explant hair follicles resulting in a dystrophic anagen pattern, characterized by condensation of the dermal papilla with disruption and abnormal keratinization of the pericortical cells of the hair matrix. These features are similar to the follicular pathology of alopecia areata (MacDonald-Hull *et al*., 1991) and the genes for these cytokines are therefore candidate genes in alopecia areata. The IL-1 system consists of at least two agonist molecules, IL-1α and IL-1β, and a structurally related receptor antagonist molecule, IL-1ra. IL-1ra binds to type 1 IL-1 receptors but does not transduce a signal, and therefore acts as a potent anti-inflammatory molecule (Dinarello, 1996).

The *IL1A*, *IL1B* and *IL1RN* (IL-1 receptor antagonist) genes are clustered within a 430-kb interval on chromosome 2q13 (Nicklin *et al*., 1994). *IL1RN* variants are associated with the severity of several inflammatory/autoimmune diseases, including ulcerative colitis (Mansfield *et al*., 1994), lichen sclerosus (Clay *et al*., 1994), psoriasis (Tarlow *et al*., 1997), myasthenia gravis (Huang *et al*., 1998), multiple sclerosis (Schrijver *et al*., 1999) and rheumatoid disease (Cox *et al*., 1999). We previously reported an association between the rare allele of the *IL1RN* VNTR and alopecia universalis, the severest form of alopecia areata (Tarlow *et al*., 1994). This has been confirmed in a recent study in which we showed a strong association with severity of alopecia areata using further markers within the *IL1RN* gene as well as the new *IL1RN* analogue, *IL1L1* (Tazi-Ahnini *et al*., in press). In the present study, we have tested alopecia areata for association with three different markers within the IL-1 cluster. Genotypes for the marker *IL1B* + 3954 are known to influence production of IL-1β (Pociot *et al*., 1992). We chose the *IL1A* + 4845 polymorphism because it has 100% linkage disequilibrium with *IL1A*-889 (Cox *et al*., 1998), which has also been shown to influence IL-1β production (Hulkkonen *et al*., 2000). There is strong linkage disequilibrium between *IL1A* + 4845 and *IL1B* + 3954. Weak linkage disequilibrium exists between *IL1B*−511 and these two markers (Cox *et al*., 1998).

Materials and methods

Patients and clinical assessment

One-hundred and sixty-five patients with alopecia areata were recruited from dermatology clinics in Sheffield, UK (aged 46.8 ± 15.4 years, female:male ratio 1.98). DNA from healthy controls was obtained from 1150 consecutive sample donations to the Trent Blood Transfusion Service, Sheffield; aged 43.9 ± 11.9 years, female:male ratio 1.03. Controls were ethnically matched to the disease population (Caucasian, northern European).

The alopecia areata patients entered into this project were managed by three consultant dermatologists (MJC, AMcD and AGM) and had been followed up for 1–6 years. The clinical diagnosis of alopecia areata was based on the presence of initially patchy alopecia with exclamation mark hairs and exclusion of other causes of alopecia (Messenger & Simpson, 1997). Detailed clinical information was obtained from each patient, including age at onset, family history of alopecia areata, type of disease (patchy alopecia, alopecia totalis and alopecia universalis), concomitant autoimmune diseases and atopy (Table 1). The clinical information was updated at follow-up visits.

DNA analysis

Genomic DNA was extracted from whole blood according to standard protocols and stored at 100 ng/μL. Three single-nucleotide polymorphisms (SNPs) in the IL-1 cluster *IL1A* (+4845), *IL1B* (+3954), *IL1B* (–511), were analysed. 25μL PCR reactions comprised 8% glycerol, 200 μM each dATP, dGTP and dCTP, 400 μM dUTP, 1.25 U AmplitaqGold (Perkin-Elmer, USA), 1.25 U Uracil-N-Glycosylase (Perkin-Elmer, USA), 5 mM $MgCl_2$ and 500–900 nM each primer. Allelic discrimination at these loci was performed using a 5′ nuclease assay (TaqMan allelic discrimination test). This is based on 5′ nuclease activity of Taq polymerase and the detection by fluorescence-resonance energy transfer (FRET) of the cleavage of two probes designed to hybridize to either allele during PCR. Double fluorescent probes were provided by ABI-PE (Forster City, CA; Warrington, UK). Probe and primer sequences, and cycling conditions are detailed in Table 2. Probes were labelled with carboxyfluorescein (FAM) and carboxy -4,7,2′,7′-tetrachlorofluorescein (TET) fluorescent dyes at the 5′ end, and the quencher carboxytetramethylrhodamine (TAMRA) at the 3′ terminus. Concentrations

Table 1 Clinical characteristics of the patient group

Clinical type	*n*
Patchy alopecia areata	92
Alopecia totalis	26
Alopecia universalis	40
Ophiasiform alopecia areata	3
Diffuse alopecia areata	4
Associated autoimmune disease	
Thyroid diseases	15
Vitiligo	3
Psoriasis	1

Table 2 Probes and PCR conditions used for genotyping *IL1A* (+4845), *IL1B* (+3954), *IL1B* (−511)

Probes and PCR conditions	
IL1A (+4845)	
Probe 1	5′-C(•FAM)AA GCC TAG GTC ATC ACC TTT TAG CTT CTT T(•TAMRA)-3′
Probe 2	5′-C(•TET)AA GCC TAG GTC AGC ACC TTT TAG CTT CTT T(•TAMRA)-3′
Forward	5′-ACC CCC TCC AGA ACT ATT TTC CCT-3′
Reverse	5′-TGT AAT GCA GCA GCC GTG AGG TAC-3′
Cycling	[95°C, 2 min] × 1; [94°C, 1 min, 65°C, 1 min, 72°C, 1 min] × 40; [94°C, 12 min, 65°C, 2 min, 72°C, 5 min] × 1
IL1B (−511)	
Probe 1	5′-C(•FAM)T CTG CCT CGG GAG CTC TCT(•TAMRA)-3′
Probe 2	5′-C(•TET)T CTG CCT CAG GAG CTC TCT(•TAMRA)-3′
Forward	5′-GTT TAG GAA TCT TCC CAC TT-3′
Reverse	5′-TGG CAT TGA TCT GGT TCA TC-3′
Cycling	[95°C, 2 min, 53°C, 1 min, 74°C, 1 min] × 2; [95°C, 1 min, 53°C, 1 min, 74°C, 1 min] × 35; [94°C, 1 min, 53°C, 1 min, 74°C, 5 min] × 3
IL1B (+3954)	
Probe 1	5′-A(•FAM)CC TAT CTT CTT TGA CAC ATG GGA TAA CGA T(•TAMRA)-3′
Probe 2	5′-A(•TET)CC TAT CTT CTT CGA CAC ATG GGA TAA CGA T(•TAMRA)-3′
Forward	5′-GCT CAG GTG TCC TCC AAG AAA TC-3′
Reverse	5′-GTG ATC GTA CAG GTG CAT CGT-3′
Cycling	[95°C, 2 min, 62°C, 1 min, 72°C, 1 min] × 2; [95°C, 1 min, 62°C, 1 min, 72°C, 1 min] × 27; [94°C, 1 min, 62°C, 1 min, 72°C, 5 min] × 3

Table 3 Allelic distribution of the three polymorphisms in the IL-1 cluster: *IL1A* (+4845), *IL1B* (+3954), *IL1B* (−511) in patients and controls. *P*-values are shown uncorrected for the number of tests performed

Marker Genotype	Alopecia areata				Controls					
	11	*12*	*22*	*n*	*11*	*12*	*22*	*n*	χ^2	*P*
IL1A+4845	90	56	17	163	539	497	109	1145	4.85	0.09
IL1B+3954	95	61	6	162	622	448	80	1150	2.89	0.23
IL1B–511	66	82	16	164	498	492	151	1141	3.28	0.19

of FAM and TET probes ranged between 20 and 50 nM and 50–350 nM, respectively, depending on the probes used. Plates were scanned in an LS50-B or a PE7200 fluorimeter (ABI/Perkin-Elmer).

In keeping with standard nomenclature, we denoted the common allele '*1*' and the rarer allele '*2*' for each polymorphic site. Homozygosity is indicated by *1,1* or *2,2* and heterozygosity by *1,2*.

Statistical analysis

Genotype distributions in cases and controls were compared using χ^2-tests on 2×3 contingency tables. Odds ratios were calculated by combining *1,2* and *2,2* genotypes against *1,1* in 2×2 tables in the overall dataset and in disease subgroups according to severity.

Results

Having checked that there was no significant deviation from the Hardy–Weinberg equilibrium for any of the markers examined (*IL1A*+4845, *IL1B*+3954 and *IL1B*−511) in patients or controls, we examined genotypic distributions of the three markers in alopecia areata patients compared to healthy controls (Table 3). There was no significant difference in the allelic distribution of the *IL1B*+3954 and *IL1B*−511 polymorphisms between patients and controls. However, there was suggestive but nonsignificant evidence of association with *IL1A*+4845 (Table 3). We then divided the patients and controls into two groups by genotype (*1,1* vs. *1,2*/*2,2*). There was borderline association between the *IL1A*+4845 polymorphism and disease in the overall dataset [OR 1.39 (1.00, 1.93)].

Subgroup analysis by disease severity suggested that this association was due mainly to the contribution of cases of mild disease (patchy AA) [OR 1.48 (0.96, 2.29)], an apparently weaker association being noted in severe disease (alopecia totalis/universalis) [OR 1.12 (0.69, 1.82)].

Discussion

In the present investigation, we have shown that there is a possible association between alopecia areata and a polymorphism of *IL1A* (+4845). The effect of this polymorphism may be more pronounced in the milder (patchy) forms of the disease. This is the opposite pattern to our previous findings with *IL1RN* in which we showed that homozygosity for the rare allele was associated with a greatly increased risk of severe disease (Tarlow *et al*., 1994). Our current observations add to the evidence suggesting genetic heterogeneity in alopecia areata and the need for larger studies with analysis of well-defined clinical subgroups of disease is emphasized.

We found no association with either of the *IL1B*+3954 polymorphisms studied in the overall dataset or in disease severity subgroups. This contrasts with the work of (Galbraith *et al*., 1999) who reported a weak association with the same marker. However, the power of the Galbraith study was low, around 41%, whereas our study had approximately 90% power to detect such an effect. It is also possible that the contrasting results may reflect different clinical characteristics in the two patient populations. On the other hand, the main finding in that study was that *IL1B* co-operates with immunoglobulin κ light chain genotypes to increase susceptibility to alopecia areata. Other investigators have reported associations with polymorphisms of *IL1B* in a range of inflammatory disorders including inflammatory bowel disease (Stokkers *et al*., 1998; Nemetz *et al*., 1999) and insulin-dependent diabetes mellitus (Pociot *et al*., 1992; Loughrey *et al*., 1998).

Recently, in our study of the *MX1* gene on chromosome 21q22.3 in alopecia areata (Tazi-Ahnini *et al*., 2000) we showed a significant association of this gene with patchy disease. The role of IL-1 cluster polymorphisms as well as *MX1* variants and HLA genotypes in the pathogenesis of alopecia areata requires further examination in large numbers of patients and especially in family studies.

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