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Investigation of the HLA-DRB1 locus in alopecia areata

To further evaluate the nature of the HLA association with alopecia areata (AA), we investigated the HLA-DRB1 locus in 161 AA patients and 165 matched controls from Belgium and Germany. HLA-DRB1 typing was performed using a recently established method that employs a combination of PCR-SSP (sequence specific priming) and PyrosequencingTM technology. No significant differences were observed for HLA allele groups DRB1 *01, *07, *08, *09, *10, *11, *13, *14, *15, and *16. HLA-DRB1*03 was found to confer a protective effect (7.5% versus 13.6%, $p = 0.011$). Additional genotyping at the allelic level revealed a significant difference in HLA-DRB1*0301 between patients and controls (6.8% versus 11.2%, $p = 0.048$). The DRB1*04 allele group was confirmed as a risk factor for the development of AA (20.8% versus 13.3%, $p = 0.012$), with the allele DRB1*0401 accounting for the greatest proportion of the effect (13.4% versus 7.3%, $p = 0.014$). Results obtained after subgrouping of the patients according to age at onset, severity and family history of the disease suggests that the genetic effects of the HLA system are strongest in familial cases of the disease.

Key words: alopecia areata, baldness, hair, HLA, major histocompatibility complex, polygenic trait, pyrosequencingTM

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Alopecia areata (AA) is a common form of hair loss affecting approximately 1-2% of the general population [1]. AA can manifest in both sexes of all age groups and the progression of hair loss is extremely variable. Classification is divided into three subtypes, depending on the degree of hair loss: the mildest form of AA involves patchy hair loss on the scalp or other areas of the body, and is also named patchy alopecia areata (patchy AA). This can either progress to alopecia totalis (AT), involving a total loss of scalp hair or to alopecia universalis (AU), involving a complete loss of scalp and body hair. The etiopathogenesis of AA is not completely understood. Alopecia areata (AA) is thought to be a tissue-specific autoimmune disease directed against the hair follicle, and may be associated with other autoimmune diseases. The mechanism of hair follicle dysfunction is immunological and is mediated by activated T-cells [2]. Familial aggregation of AA has been reported by numerous studies [3-6]. In the first family study of AA to obtain

information directly from the relatives of AA patients, we recently found that 21.8% of 206 AA patients have at least one first-degree relative with AA and 34.0% have at least one first- or second-degree relative with AA [6]. Among first-degree relatives, the lifetime risk is estimated at 7.8% in parents, 7.1% in sibs and 5.7% in children. The sibling risk ratio was calculated to be 4.2, which is in the range found for other common disorders with a multifactorial etiology.

Various functional candidates have been tested as possible disease susceptibility genes [7-10] but it is only in the case of the major histocompatibility complex (HLA) region on chromosome 6p21, that evidence has been obtained from a large number of independent studies. The HLA alleles found most consistently to be involved include alleles of the HLA class II DRB1 system [11-23]. Despite these studies, however, a specific gene defect for AA has not yet been identified in the HLA region. Further studies of AA patients that define the HLA region in more detail are needed, an

approach that might finally lead to the identification of a causal genetic variant. In this study we aimed to investigate the relation between AA and the HLA-DRB1 locus in a Belgian-German sample of AA patients and various subgroups.

Materials and methods

Patients

161 unrelated patients with AA (100 women and 61 men) aged 5-78 years (mean age 53) were included in this study. The patients were recruited from the outpatient hair clinics of two Departments of Dermatology, the University Hospitals at Antwerp (Belgium) and Düsseldorf (Germany). Patients from Düsseldorf (n = 20) represent a group of newly diagnosed patients, while the sample from Antwerp (n = 141) was collected retrospectively. Clinical data of all patients were obtained, including age at onset and familial occurrence. The severity of alopecia was assessed according to the alopecia areata investigational assessment guidelines [24] and patients were categorized as having either patchy alopecia (S₁-S₄), alopecia totalis (AT), alopecia totalis/universalis (AT/AU), or alopecia universalis (AU). Patchy alopecia includes the stages S₁ (less than 25% hair loss) to S₄ (75-99% hair loss), AT was defined as 100% scalp hair loss without loss of body hair, AT/AU was defined as 100% scalp hair loss with variable loss of body hair, AU was defined as 100% loss of both scalp and body hair. The assessment of severity is based on a lifetime perspective and is according to the most severe episode ever experienced. Inherent to this severity assignment is that assignment of patients to the less severely affected subgroups is not certain since the patients may develop more severe forms of the disease later in life.

74 patients had patchy AA, 12 AT, 2 AT/AU and 73 had AU. The patients were grouped into two categories, *i.e.* mild AA (all patients with patchy AA) and severe AA (combining the patients with AT, AT/AU and AU). There was a family history of AA in 44/161 patients (27.3%), defined as having at least one first or second degree relative with AA. Age at onset was defined as the age when patchy hair loss was first noticed. Early onset alopecia (age at onset ≤ 20 years) occurred in 79/161 patients (49.1%), late onset alopecia (age at onset > 20 years) was observed in 82/161 patients (50.9%). All patients gave written informed consent for the genetic studies, and the ethics committees of both institutions approved the study.

The control group was comprised of 165 healthy unrelated sex and age matched blood donors of German descent. Blood donors were not specifically screened for the absence of AA as this would have little impact on the power of a case-control study when the disease studied has a population prevalence of approximately 1-2% as reported for AA [25].

Typing methods and statistical analysis

HLA-DRB1 typing was performed using a combination of SSP (sequence-specific-priming) and a HLA-DRB1 specific Pyrosequencing™ approach as described elsewhere [26]. In a first step, the PCR-SSP typing allows determination of DRB1 group specificity in patients and in controls. In a second step, samples with genotypes belonging to

Table 1. Frequency of HLA-DRB1 alleles in all patients with alopecia areata compared with controls

HLA-DRB1	Alleles in Controls	%	Alleles in Patients	%	p-value
*01	39	11.8	41	12.7	0.744
*15, *16	58	17.6	58	18.0	0.913
*08, *12	20	6.1	16	5.0	0.529
*03, *11, *13, *14	140	42.4	116	36.0	0.082
thereof *03	45	13.6	24	7.5	0.011
*0301	37	11.2	22	6.8	0.048
*0302	1	0.3	0	0	1.000
*03XX ^a	7		2		
thereof *11	45	13.6	38	11.8	0.464
*04	44	13.3	67	20.8	0.012
*0401	24	7.3	43	13.4	0.014
*0402	1	0.3	1	0.3	1.000
*0403	5	1.5	0	0	0.076
*0404	6	1.8	3	0.9	0.520
*0405	1	0.3	2	0.6	0.987
*0407	1	0.3	3	0.9	0.603
*0408	1	0.3	3	0.9	0.603
*04XX ^a	5		12		
*07	27	8.2	21	6.5	0.405
*09	0	0	1	0.3	0.993
*10	2	0.6	2	0.6	1.000
alleles (2n)	330		322		

^a DRB1*03- and DRB1*04-subtyping failed because of insufficient amount of DNA.

those allele groups in which the allele frequencies in patients clearly differed from controls were analyzed at a high resolution level by pyrosequencing. Pyrosequencing was carried out on a PSQ96 (Pyrosequencing, Uppsala, Sweden) according to the protocols of the supplier and using the before mentioned sequencing strategy. The pyrograms were evaluated "by hand" and the resulting sequences were compared with the HLA-DRB1 allele sequences in the database (IMGT/HLA data base, <http://www.ebi.ac.uk/imgt/hla/>). Statistical analysis was performed by two-by-two contingency tables. Fisher's exact two-tailed test was calculated. When significant p-values were achieved the odds ratio and 95% confidence intervals were calculated. In calculating the frequencies of HLA alleles, patients with only a single detected allele were assumed to be homozygous for that allele. A significance level of p > 0.05 was assumed for all statistical tests.

Results

In our study, the DRB1 locus was examined in a sample of 161 AA patients as compared to a sex- and age matched control sample using a combination of PCR-SSP and Pyrosequencing™.

Table 1 summarizes the results of the HLA-DRB1 allele frequencies obtained in AA patients and controls. The frequency of the HLA-DRB1*03 allele was significantly decreased in patients (7.5%) as compared to controls (13.6%) (p = 0.011, odds ratio 0.510, 95% CI 0.293-0.884). Further

Table 2. Frequency of HLA-DRB1 alleles according to severity, age at onset and familiarity (%)

HLA-DRB1	Mild AA	Severe AA	P-value	Early-onset AA	Late-onset AA	P-value	Familial AA	Sporadic AA	P-value
alleles (2n)	148	174		158	164		88	234	
*01	14.9	11.1	0.317	14.7	11.1	0.403	10.2	13.7	0.459
*15, *16	21.6	15.1	0.146	19.3	16.9	0.565	18.2	18.0	1.000
*08, *12	6.8	3.5	0.204	4.7	5.2	1.000	2.3	6.0	0.252
*03, *11, *13, *14	32.4	39.0	0.294	30.0	41.3	0.037	34.1	36.8	0.697
thereof *03	8.1	7.0	0.678	6.0	8.7	0.400	2.3	10.3	0.020
*0301	6.8	7.0	1.000	6.0	7.6	0.661	2.3	8.6	0.049
*0302	0.0	0.0	1.000	0.0	0.0	1.000	0.0	0.0	1.000
thereof *11	8.8	14.5	0.165	10.7	12.8	0.606	10.2	12.4	0.700
*04	16.9	24.4	0.130	22.7	19.2	0.492	26.1	18.8	0.166
*0401	9.5	16.9	0.071	16.0	11.1	0.250	18.2	11.5	0.141
*0402	0.0	0.6	1.000	0.0	0.6	1.000	1.1	0.0	0.273
*0403	0.0	0.0	1.000	0.0	0.0	1.000	0.0	0.0	1.000
*0404	0.7	1.7	0.628	0.7	1.7	0.626	1.1	1.3	1.000
*0405	0.7	0.6	1.000	0.7	0.6	1.000	0.0	0.9	1.000
*0407	0.7	1.2	1.000	1.3	0.6	0.600	1.1	0.9	1.000
*0408	2.0	0.0	0.096	0.7	1.2	1.000	0.0	1.3	0.565
*07	6.8	6.4	1.000	7.3	5.8	0.654	9.1	5.6	0.310
*09	0.0	0.6	1.000	0.7	0.0	0.466	0.0	0.4	1.000
*10	0.7	0.6	1.000	0.7	0.6	1.000	0.0	0.9	1.000

subgrouping of DRB1*03 alleles demonstrated that *0301 appears to be the mainly responsible allele (6.8% versus 11.2%, $p = 0.048$, odds ratio 0.581, 95% CI 0.323-1.041). The frequency of the DRB1*04 allele was significantly increased in AA patients (20.8%) when compared to controls (13.3%) ($p = 0.012$, odds ratio 1.708, 95% CI 1.104-2.645). Subdivision of the *04 group revealed that the *0401 allele was the only allele to achieve significance (13.4% versus 7.3%, $p = 0.014$, odds ratio 1.965, 95% CI 1.129-3.435) and accounted for the greatest proportion of the effect.

The other DRB1 allele groups that have been tested (*01, *07, *08, *09, *10, *11, *13, *14, *15, *16) failed to achieve statistical significance.

Table 2 divides the DRB1 allele frequencies according to severity, age at onset and familiarity. In terms of severity, we found no significant differences between patients and controls for the HLA-DRB1 alleles tested. However, when we also take non-significant trends into account, the risk allele *0401 seems to have a more pronounced effect in severe cases than in mild cases of AA (16.9% versus 9.5%, $p = 0.071$). When we compared patients with an early onset of alopecia (≤ 20 years) to patients with a later age at onset (> 20 years), only one comparison reached statistical significance (group *03, *11, *13, *14: 30.0% versus 41.3%, $p = 0.037$, odds ratio 1.640, 95% CI 1.006-2.677). Further subdivision of alleles, however, failed to implicate individual alleles.

In patients with a positive family history of AA, we observed a significant difference in HLA-DRB1 allele frequencies for *03 and *0301 when compared to patients without a family history (10.3% vs. 2.3%, $p = 0.020$, odds ratio 4.914, 95% CI 1.092-30.778 and 8.6% vs. 2.3%, $p = 0.049$, odds ratio 4.019, 95% CI 0.879-25.489). A similar trend for a stronger effect in familial cases was observed

with the *0401 allele (18.2% in familial cases versus 11.5% in sporadic cases of AA). This comparison, however, did not reach statistical significance ($p = 0.141$).

Discussion

Due to the pattern of familial occurrence in AA, there is a strong assumption that the inheritance pattern of AA is that of a complex genetic trait. Although an autoimmune pathomechanism for AA has been suggested, the precise etiology is unknown, and no autoantigen or causative gene has been identified so far. However, an association between alopecia areata and HLA alleles, especially HLA-DRB1, has been described in the literature [11-23]. A comparison of several studies with samples of diverse ethnic backgrounds reveals differences in the critical allele as well as varying allele frequencies between populations. Since no specific gene variant has yet been identified as a cause of AA despite positive associations, there is a need to identify associated alleles in each particular population.

Our study, which concentrated on the HLA-DRB1 locus in a Belgian-German sample, showed a significant increase in the appearance of DRB1*04 alleles and a significant decrease in the appearance of DRB1*03 alleles in patients with AA, as discussed in more detail below.

Our study confirms that DRB1*03 is a protective factor against the development of AA and suggests that it is the DRB1*0301 allele that confers the major portion of this effect. A protective effect of DRB1*03 has been suggested previously, based on the investigation of Polish and Turkish patients [23, 22]. Due to the small sample sizes of these previous studies, with 52 patients having been included in the Polish study and 65 patients in the Turkish study, the results from their further subgrouping of patients were not

conclusive. In our study, where we investigated a substantially larger sample of patients, we observed no significant frequency differences in patients subgrouped according to severity of disease and age of onset. Smaller effects still remain possible, however, and require enlargement of samples. Interestingly, we observed a significant difference ($P = 0.02$) in DRB1*03 frequency between familial (2.3%) and sporadic cases (10.3%), suggesting that the protective effect of DRB1*03 is mainly present in patients with a genetic background of the disease while it is very small in non-familial cases. We regard our method of assessing familiarity in our patients as relatively sensitive since we directly interviewed all available first-degree relatives and obtained information about second-degree relatives through interviews with patients and first-degree relatives. The high sensitivity in identifying familial cases is illustrated by the percentage of familial cases (27.3%) found in our sample which is in the upper range of rates reported in the literature [3, 5].

Our study also provides strong support for a risk conferring role of DRB1*04 in the European population. Similar results have been reported for North American white, UK white, Danish and Turkish patients [12, 14-16, 18-20, 22]. It has been previously suggested that the effect of DRB1*04 is restricted to long-standing disease [18, 19]. We did not test this since we had not obtained this information from our patients in a standardized manner. A long-standing course of disease is, however, associated with early-onset and a severe clinical expression of the phenotype. Subdividing our patients, we observed differences that were in the expected direction. The observed tendencies, however, did not reach thresholds for significance, highlighting the importance of a large sample size in addressing differential effects between subgroups of patients with sufficient power. In our study, we further discriminated between subclasses of DRB1*04. The most prevalent allele, DRB1*0401, seems to be responsible for the effect of DRB1*04, confirming results obtained in a North American White population [18, 19].

To our knowledge, this is the first study investigating HLA class II alleles in Belgian-German patients with AA. We have been able to confirm the protective effect of DRB1*03 and the predisposing effect of DRB1*04 in the development of AA. Additional genotyping at the allelic level has attributed the effect of DRB1*03 to DRB1*0301 and confirmed the effect of DRB1*0401 in the DRB1*04 group. On the basis of results from the subgrouping of patients, we suggest that the protective effect of DRB1*03 is mainly conferred by patients with a positive family history of the disease. This finding underscores the importance of documenting family history in genetic research into AA. ■

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