

Alopecia Areata in Families: Association with the HLA Locus

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Alopecia areata (AA) is a T cell mediated disease directed against hair follicles that results in bald patches. It can range in severity from patchy (AA), to total scalp hair loss (alopecia totalis; AT) or body hair loss (alopecia universalis; AU). We have previously shown that HLA-DR4 and DR11 as well as HLA-DQ*03 alleles are increased in unrelated AA patients compared with controls. To study whether class II HLA alleles are linked to AA, we investigated 81 extended families that included 192 AA patients, including 89 with AT or AU. We also performed the transmission disequilibrium test (TDT) in 143 nuclear families. Results showed an association between alleles of HLA-DQB ($p=0.014$) and HLA-

DR ($p=0.010$). We also performed linkage analysis in 75 families whose members' genomic DNA were available for HLA typing. Results from this analysis support linkage between AA and class II loci with a maximal LOD score of 2.42 to HLA-DQB at 5% recombination, and with a maximal LOD score of 2.34 to HLA-DR at 0% recombination. There was an increased incidence of atopic dermatitis and autoimmune thyroiditis in families. AA appears to be a class II HLA restricted organ specific immune response to the hair follicle. Key words: autoimmune disease/baldness/hair/HLA disease association/linkage. Journal of Investigative Dermatology Symposium Proceedings 4:220-223, 1999

Alopecia areata (AA) is a T cell mediated immune response directed at hair follicles, and may be another organ specific autoimmune disease, like diabetes, in which there is failure of self tolerance (Sinha *et al*, 1990; Kouskoff *et al*, 1996). The immune infiltrate results in abnormal keratinization and hair breakage within the follicle and is generally present throughout the scalp (Nutbrown *et al*, 1995; Ghersetich *et al*, 1996); however, the severity of the clinical phenotype varies from discrete patchy hair loss in one or several areas to total loss of scalp hair (alopecia totalis; AT) and body hair (alopecia universalis; AU) (Hordinsky, 1991; Price, 1991; Kalish *et al*, 1992; Shapiro, 1993). The overall incidence of AA in one county was calculated to be 20.2 per 100 000 person years with a lifetime risk of 1.7%, and the rate did not vary with gender or age (Safavi *et al*, 1995). Although most cases of AA are sporadic, a genetic basis for AA has been suggested. It occurs in families in 20% of individuals and may be associated with other autoimmune diseases (Cunliffe *et al*, 1969; Valsecchi *et al*, 1985; Zlotogorshi *et al*, 1990; Shellow *et al*, 1992; van der Steen *et al*, 1992; Shapiro, 1993). AA has been reported in sets of identical twins (Turnacliffe, 1931; Cole and Herzlinger, 1984). We have recently found that the concordance rate is 55%, similar to that of psoriasis, suggesting that an environmental trigger may be important in addition to genes (Jackow *et al*, 1998). Two inbred rodent models develop hair loss and T cell infiltration similar to AA: the aging C3H/HeJ mouse

and the Dundee experimental bald rat (DEBR) (Michie *et al*, 1991; Sundberg *et al*, 1994, 1995; Zhang and Oliver, 1994). In the DEBR rat, depletion of the CD8⁺ T cell population restores hair growth and the alopecia responds to immunosuppressive therapies (Mitsuya *et al*, 1985).

A number of organ specific autoimmune diseases have been identified, including diabetes, autoimmune thyroiditis, psoriasis, inflammatory bowel disease, rheumatoid arthritis, and systemic lupus (Todd *et al*, 1988a; Gregersen, 1989). Each is characterized by associations with specific alleles of the HLA or major histocompatibility complex (Todd *et al*, 1988a; Garchon and Bach, 1991) (**Table II**). Genes encoding the polymorphic HLA proteins reside on human chromosome 6p21 (Trowsdale *et al*, 1991). HLA proteins are expressed on the cell surface and function in self-recognition and in presentation of peptide moieties to T cells (Todd *et al*, 1988a). HLA Class I (HLA-A, B, C) chains form complexes with B2 microglobulin and are found on the surface of all nucleated cells. Class I antigens present peptides (usually viral derived) to CD8 or suppressor T cells. Class 2 HLA molecules are heterodimers of alpha and beta chains that bind processed antigen and present to CD4 or helper T cells, resulting in help for the production of antibodies. The amino acid sequences of the hypervariable regions of the HLA chains determine the affinity of peptide binding and thus may restrict the type of immune reactions a host can mount in the face of antigenic challenge (Todd *et al*, 1988a; Gregersen, 1989; Sinha *et al*, 1990). Polymorphic amino acid residues that may be shared by several class II alleles (shared epitopes) may confer susceptibility to rheumatoid arthritis, Type I diabetes mellitus, and pemphigus vulgaris (Todd *et al*, 1988b; Gregersen, 1989; Sinha *et al*, 1990). In different ethnic populations, different HLA alleles may be associated with the same disease, yet there remains a common sequence across ethnic lines that confers disease susceptibility (Todd *et al*, 1989). The "dosage" (heterozygosity *versus* homozygosity) of the

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Abbreviations: AA, alopecia areata; AU, alopecia universalis; AT, alopecia totalis.

disease-associated sequence influences susceptibility to and/or severity of the autoimmune disease (Sheehy *et al*, 1989; Todd *et al*, 1989; Weyland *et al*, 1992).

In association studies conducted in unrelated individuals with AA, there are significant HLA associations reported for both class I and class II HLA alleles (**Table I**). An HLA-class I association with HLA-B12 (RR = 5.41) has been reported for patchy AA (Kianto *et al*, 1977). Other class I MHC alleles, HLA-B18, B13, and B27, were first noted to be increased in unrelated patients with AA from different groups (Kuntz *et al*, 1977; Hacham-Zadeh *et al*, 1981; Minev *et al*, 1983; Averbakh and Pospelo, 1986). More recent studies, performed by sequence based oligonucleotide typing implicate HLA-DR5, now the HLA-D11 allele, and found that the subtype DRB*1104 is highly associated with early onset and severe patchy AA (Welsh *et al*, 1994; Colombe *et al*, 1995). In addition, 92% of patients with AU/AT and 80% of all AA patients carry one of the DQB*03 alleles (Welsh *et al*, 1994; Colombe *et al*, 1995). The combination of HLA-DR4 and DR5 was also increased among AA patients, whereas the presence of HLA-DRB3-w52a was significantly decreased (Duvic *et al*, 1991; Welsh *et al*, 1994). An association of AA with DP alleles (Odum *et al*, 1990) could not be confirmed (Welsh *et al*, 1994).

In order to study the genes that are associated with AA, family studies must be conducted. These studies depend on showing an association between genetic variations (markers, alleles) and the trait (AA). If a gene causes AA, then either the gene or a marker located very close to the gene on the same chromosome should track together. This is called genetic linkage and is based on the fact that the closer two pieces of DNA are to one another, the less frequently they will be separated during the process of crossing over or recombination that occurs randomly during mitosis. This paper presents preliminary studies of class II HLA association and linkage in families and sibling pairs with AA. Diabetes currently has 15 associated genes, including HLA, based on a genome wide search (Davies *et al*, 1994), and hence the analysis of the immunogenetic basis for AA has just started.

MATERIALS AND METHODS

Subjects Cases were identified from registrants at the Dermatology Clinics of the University of Texas Health Science Center at Houston,

including the clinics at the Houston Medical Center, Herman Professional Building, Lyndon B. Johnson General Hospital and the M.D. Anderson Cancer Center. Affected individuals also self-referred themselves through information obtained from the National Alopecia Areata Foundation and through the World Wide Web page for Alopecia Areata (<http://weber.u.washington.edu/~dvictor>) maintained by Dan Victor at the University of Washington. The cases were contacted by telephone, the study explained in detail, and consents to participate were requested from all family members. Willing participants signed an informed consent form for collection of blood samples and data. A questionnaire to determine the severity of AA and other associated factors following the guidelines of the National Alopecia Areata Foundation was completed. Diagnosis of AA was confirmed by the subject's dermatologist and a pedigree of the family was obtained from the proband.

Preparation of the DNA and HLA typing The DNA was extracted from peripheral blood using a standard protocol according to the manufacturer's instructions (Stratagene, La Jolla, CA). One microgram of extracted DNA was used for the amplification of the DRB1 and DQB1 loci according to the Eleven HLA International Workshop by polymerase chain reaction and Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The DRB1 and DQB1 generic primers used were: for DRB1, DRB ampA 5'-CCCCACAGCACGTTTCTTG-3' and DRB ampB 5'-CATGTGCACTGTGAAGCTC-3'; and for DQB1, DQB ampA 5'-CATGTGCTACTTCACCAACGG-3' and DQB ampB 5'-CTGGTAGTTGTTGTGTCTGCACAC-3'. The conditions for the amplification were the ones recommended by the Eleven International Workshop with an annealing temperature of 55°C for 1 min for DRB1 and 30s for DQB1. The amplified DNA was transferred to Zetabind Membranes (Cuno, Meridian, CT) by DOT-Blotting and hybridized to specific 5 γ ATP-P32 end-labeled oligonucleotide probes (Amersham, Arlington, IL) at 54°C for 2 h. The radioactive probes were not used at less than 1.106 cpm per ml of hybridization. The membranes were washed in solutions containing 3 M tetramethylammonium chloride and 2 \times SSPE and finally exposed to X-Ray films overnight at -70°C.

Statistical analysis Standard Chi² or Fisher's exact test was performed to test for association between frequencies of autoimmune diseases and AA.

We performed tests for association between alleles at specific candidate loci and risk of AA. To ensure that we did not identify spurious associations, we used the transmission disequilibrium test (TDT) (Spielman *et al*, 1993). The TDT was introduced as a test for linkage between a complex disease and a genetic marker. This test is carried out with data on transmission of marker alleles from parents heterozygous for the marker to affected offspring. The TDT is a valid test for linkage and association, even then the association is caused by population subdivision and admixture (Ewens and Spielman, 1995). We applied the marginal homogeneity test, which allows for the multiple testing problem when considering multiple alleles at each locus. We used permutation under the null hypothesis to obtain an empirical p-value to allow for decreased effects from rare alleles. The TDT is performed using a SAS macro developed by the Department of Epidemiology, M.D. Anderson Cancer Center.

For linkage analysis, PAP (Pedigree Analysis Package) was chosen for its flexibility of modeling for linkage studies in these patients (Hasstedt, 1994). The association between AA and HLA class II alleles was included as a part of the analysis by assuming that the carrier probabilities varied as a function of the HLA class II alleles in each founding parent. This approach for linkage analysis improves the power to detect genetic linkage. Although associations are included in the analysis, these are only important in assigning the probability that a founding individual is a carrier of AA-predisposing allele, conditional on his or her class II alleles. For children, no association between class II alleles and susceptibility to AA is made, so that there is no bias in favor of detecting a false linkage under this modeling scheme.

Table I. HLA associations reported with AA

Author	No. patients	Ethnic group	Association
Kianto	47	Finnish	HLA-B12
Hacham-Zadek	46	Jewish	HLA-B18
Frenz	22	Dannish	HLA-DR4 & 5
Averbakh	72	Russian	HLA-B13,27 HLA-Cw1, w3 HLA-DR7
Orecchia	127	Italian	HLA-DR5
Duvic	88	American	HLA-DR4, DR5 HLA-DQw7,8
Welsh	85	American	HLA-DRB1*1104 HLA-DQB1*03

Table II. Incidence of autoimmune diseases in individuals who have either AA or AU/AT

Autoimmune diseases	Unaffected	All affected	AA	AU/AT
Vitiligo		9 (5%)	6 (6%) (p = 0.05)	3 (3%)
Graves' Disease	3 (1%)	4 (2%)	4 (1%) (p = 0.07)	0 (0%)
Hashimoto's thyroiditis	10 (3%)	16 (8%) (p = 0.01)	7 (7%)	9 (10%) (p = 0.01)
Diabetes mellitus	8 (3%)	7 (4%)	3 (3%)	4 (4%)
Atopic dermatitis/eczema	8 (3%)	37 (19%) (p = 0.001)	13 (13%) (p = 0.001)	24 (27%) (p = 0.001)
Psoriasis	3 (1%)	2 (1%)	2 (2%)	0 (0%)
Total	305	192	103	89

A segregation analysis was done and the penetrance value was determined for the linkage studies. Based on this analysis we assumed that the penetrance of AA was 16% and the population prevalence was 0.1%.

RESULTS

Patient data Over a period of 8 y, 136 families with at least two affected members have been identified. From the material in the questionnaires completed by each participant we obtained information regarding the prevalence of other autoimmune diseases associated with AA. HLA typing was completed in 497 responding individuals among 81 extended families. These included 192 individuals affected with AA, of which 89 had the more severe forms of alopecia totalis (AT) or universalis (AU).

Among 13 sets of identical twins, six male sets were concordant for AA or AT/AU and seven sets were discordant, including four males and three females, as reported separately (Jackow *et al*, 1998).

When we examined the sex of individuals from 72 affected sib-pairs with AA or AT/AU, the incidence was similar among sisters and brothers. Brother pairs represented 31% of sib-pairs, 36% were females, and 33% were brother/sister pairs. Among brother pairs and sister pairs, the incidence of AA/AA, AA/AU, and AU/AU was equally divided. Among brother-sister sib-pairs, in only 25% did both have AU.

Association between AA and other autoimmune diseases In AA families other autoimmune diseases were commonly reported. These included vitiligo, autoimmune thyroiditis including Hashimoto's thyroiditis and Graves' Disease, diabetes mellitus, atopic dermatitis, eczema, and psoriasis. The incidence of each is shown in **Table II**. This analysis was performed from data collected on all affected individuals with AA or AT/AU and their unaffected family members who completed the questionnaires.

Among all AA or AT/AU individuals, as well as the subset with the more severe expression, AT/AU, there were significant associations with atopic dermatitis, and Hashimoto's thyroiditis (**Table II**). Individuals who have the milder phenotype, AA, also had a significant association with atopic dermatitis as well as borderline associations with vitiligo and Graves' Disease (**Table II**).

If we considered the families as units rather than looking at individuals, 19 of 82 (23%) of the families had a proband with both AA and atopic dermatitis. No association was seen between atopic dermatitis and AA using TdT analysis in families.

HLA-DR4 and 6 are significantly associated with AA From the 81 extended families who had completed HLA typing, there were 143 nuclear families. TDT method was applied to these families. The results showed an association between the alleles of HLA-DQB ($p = 0.009$) and HLA-DR ($p = 0.008$). We subsequently evaluated effects from each allele of HLA-DQ and HLA-DR to identify specific associations with disease. This analysis showed a significant association between the AA trait and DQB1*302 ($p = 0.0002$), DQB1*601 ($p = 0.041$), DQB1*603 ($p = 0.027$), HLA-DR4 ($p = 0.005$), and HLA-DR6 ($p = 0.005$).

The HLA class II loci are linked to AA Linkage analysis also supports genetic linkage between AA and HLA class II loci with a maximal LOD score of 2.42 for HLA-DQB1 at 5% recombination, and with a maximal LOD score of 2.34 for HLA-DR at 0% recombination.

DISCUSSION

Disease association studies, conducted in unrelated individuals with AA, have shown that there are significant HLA associations between AA and alleles at both the HLA class I and the HLA class II loci (Kianto *et al*, 1977; Kuntz *et al*, 1977; Hacham-Zadeh *et al*, 1981; Averbakh and Pospelo, 1986; Frenz *et al*, 1986; Duvic *et al*, 1991; Welsh *et al*, 1994; Colombe *et al*, 1995). Genetic association studies assume that the disease locus and the selected markers will be tightly linked in the population. As a result, certain markers

would be associated with AA and one would expect a significant difference in marker allele frequencies between affected and unaffected individuals. The strongest HLA associations reported to date have been with HLA-DR5 (HLA-DRB*1104), especially in a subset of patients who presented in childhood with severe, patchy AA that was persistent (Welsh *et al*, 1994; Colombe *et al*, 1995). The HLA-DQB1 genes in linkage disequilibrium with HLA-Dw11 were also significantly increased (Morling *et al*, 1991; Welsh *et al*, 1994; Colombe *et al*, 1995). Genes that may protect against manifesting AA have also been suggested, e.g., HLA-DRw52_a (Duvic *et al*, 1991).

This study investigated whether AA was associated or linked to genetic markers (HLA class II DR and DQ alleles) in families. Data on the frequency of other autoimmune diseases in families with AA were also collected and analyzed. To ensure that we do not identify spurious associations, we used the transmission disequilibrium test (Ewens and Spielman, 1995; Terwilliger, 1995). This test compares alleles transmitted to affected children to those alleles in their parents that were not transmitted. Therefore, one would expect in an AA population, a higher proportion of one or more alleles in the HLA class II DR and DQB1 genes. In our study, we observed a significant association between three alleles of DQB1 (*302, *601, and 603) and two alleles of HLA-DR (DR4 and DR6). The most significant association with HLA-DQB 0*302 ($p = 0.0002$) was not surprising, as it has previously been reported in unrelated association studies and is in linkage disequilibrium with both HLA-DR4 and DR5 alleles (Welsh *et al*, 1994; Colombe *et al*, 1995). Although HLA-DR4 was associated in this family study, no significant association was observed with the associated HLA-DR5 allele seen in unrelated individuals.

Linkage analyses provide other means of testing genetic hypotheses. For the linkage analysis, the association between AA and HLA class II alleles was included as a part of the analysis by assuming that the carrier probabilities varied as a function of the HLA class II alleles in each founding parent. This approach for linkage analysis improves the power to detect genetic linkage. In our study, we observe evidence of linkage for HLA-DQB1 and HLA-DR; however, the LOD scores were in the range of 2.5 and generally a LOD score of 3.0 or greater is required to demonstrate significant linkage. In a previous analysis of the first 29 families collected, we had shown a positive LOD score of 1.05 for HLA-DQ with a recombination fraction of 0.07 (Duvic *et al*, 1995). This previous analysis was performed without considering the association between AA and HLA class II alleles. This method is therefore less powerful.

Other autoimmune diseases, including atopy, thyroiditis, diabetes, and vitiligo, were found in individuals from AA families. Thyroiditis was significantly higher in the affected individuals than in unaffected individuals, but this association has previously been well established. There were significant differences seen in the incidence of atopic dermatitis between unaffected individuals (3%), versus those affected with AA (13%) or those with more severe forms AT/AU (27%). There was no association by TdT between AA and atopic dermatitis in families (de Andrade and Duvic, unpublished data). There is some evidence that atopy is also a multigenetic inherited phenotype with an environmental trigger (McNally *et al*, 1998). There is a bias in data derived from families. When only the proband was considered from each family, 23% had both atopic dermatitis and AA. Previous family studies have suggested that atopy is commonly seen in families where AA is present and have suggested that it is associated with a more severe phenotype, i.e., AU (Shellow *et al*, 1992; De Weert *et al*, 1984). The true incidence of atopic dermatitis is unknown and has been reported to be anywhere from 7% to 31% based on several epidemiologic studies (Leung *et al*, 1999; Nanda *et al*, 1999). Thus, it would appear that the unaffected individuals within AA families may have a less than expected incidence of AA (3%) and that the incidence in individuals is either normal or slightly higher than in the general population.

In conclusion, these data support both association and linkage between AA and HLA class II genes. AA families appear to have other autoimmune diseases and there may be a significant increase in atopic dermatitis. AA is hypothesized to be an organ specific autoimmune reaction caused by the interactions of multiple genes, each of which contributes to the eventual phenotype (Todd *et al*, 1989; Davies *et al*, 1994). Identical twins have a concordance rate of 55% and the severity of the phenotype may differ, with the first affected most severe (Jackow *et al*, 1998). The complexity and the incomplete penetrance of the AA, as well as twin studies, suggest that other genes in addition to HLA alleles, as well as environmental factors or triggers, may be involved in its expression.

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