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Genomewide Scan for Linkage Reveals Evidence of Several Susceptibility Loci for Alopecia Areata

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Alopecia areata (AA) is a genetically determined, immune-mediated disorder of the hair follicle that affects 1%–2% of the U.S. population. It is defined by a spectrum of severity that ranges from patchy localized hair loss on the scalp to the complete absence of hair everywhere on the body. In an effort to define the genetic basis of AA, we performed a genomewide search for linkage in 20 families with AA consisting of 102 affected and 118 unaffected individuals from the United States and Israel. Our analysis revealed evidence of at least four susceptibility loci on chromosomes 6, 10, 16 and 18, by use of several different statistical approaches. Fine-mapping analysis with additional families yielded a maximum multipoint LOD score of 3.93 on chromosome 18, a two-point affected sib pair (ASP) LOD score of 3.11 on chromosome 16, several ASP LOD scores >2.00 on chromosome 6q, and a haplotype-based relative risk LOD of 2.00 on chromosome 6p (in the major histocompatibility complex locus). Our findings confirm previous studies of association of the human leukocyte antigen locus with human AA, as well as the C3H-HeJ mouse model for AA. Interestingly, the major loci on chromosomes 16 and 18 coincide with loci for psoriasis reported elsewhere. These results suggest that these regions may harbor gene(s) involved in a number of different skin and hair disorders.

Alopecia areata (AA [MIM 104000]) is one of the most common human autoimmune diseases, with a lifetime risk of ~2%.[1–3] In the United States, >4.5 million people are affected with AA (National Alopecia Areata Foundation), which affects both sexes at all ages and in all ethnic groups. It is characterized by patchy hair loss on the scalp (fig. 1*A* and 1*B*), which can eventually involve the entire scalp, a condition known as "alopecia totalis," or the entire body, a condition known as "alopecia universalis" (fig. 1*C*). The onset of the disease can be sudden, its progression is unpredictable, and it can be recurrent throughout life. Reports in the literature of overnight whitening of the hair represent the abrupt onset of AA, since it preferentially targets pigmented hairs, leaving only white hairs behind (fig. 1*D*).[4–7] The pathology of alopecia extends far beyond the physical aspects of hair loss, and it can have a deeply disturbing psychological impact on affected individuals.[8–10] Even among patients with minimal hair loss from AA, the loss carries significant emotional and psychological meaning that not only pertains to hair but also has a profound impact on an individual's quality of life, ability to function in society, and preservation of self-esteem, and it can lead to profound psychological disturbances.[8,10]

Despite its high prevalence and the inherent visibility of the phenotype, the pathogenesis of AA is poorly understood, and there has been significant debate about whether the primary defect is in the hair follicle, the immune response, or both. Because of the presence of a peribulbar lymphocytic infiltrate in the scalp biopsy specimens of affected patients and the positive response of the disease to steroid treatment, an autoimmune mechanism has been postulated for many years, although an autoantigen has not yet been identified.[11–16] More recently, attempts to arrive at a unified hypothesis have led to the description of AA as a tissue-specific autoimmune disease of the hair follicle.[1] The hair follicle is an immune-privileged site, with low levels of major histocompatibility complex (MHC) expression, and the emerging view is that AA represents a breakdown in immune privilege and the subsequent destruction of the hair follicle by T-lymphocytes. Thus, AA can be considered a genetically determined, immune-mediated disorder, which therefore should be amenable to genetic linkage studies.

It is now generally accepted that AA fits the paradigm of a complex or multifactorial genetic trait, on the basis of several lines of evidence: (i) its prevalence in the population (~2%),[17,18] (ii) concordance in twins (55%),[19] (iii) a Gaussian distribution of severity,[2] (iv) a 10-fold increased

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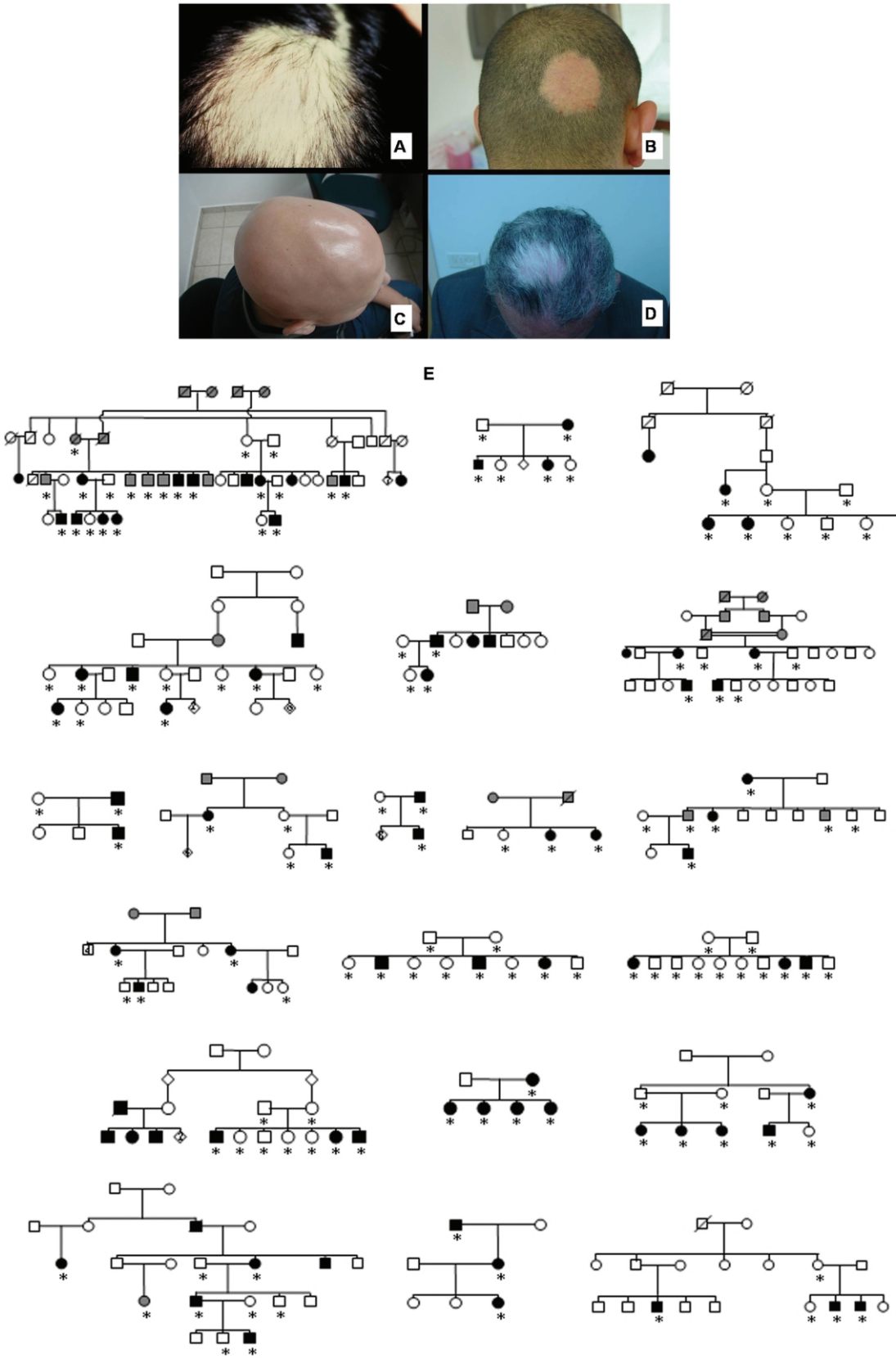

Figure 1. Clinical presentation of AA. *A* and *B,* AA appearing as well-circumscribed patches of hair loss on the scalp. *C,* Alopecia universalis, the complete form of AA, which leads to absence of all hair on the body. *D,* AA with selective loss of pigmented hair, with a patch of white hair left behind. *E,* Representative examples of pedigree structures of the families with AA. Participating family members are indicated by an asterisk (*).

Table 1. Modes of Inheritance and Penetrance Values Used to Compute MAXHLOD

Genetic Model[a]	Penetrances[b]			Disease-Allele Frequency
	Pr(aff/++)	Pr(aff/+d)	Pr(aff/dd)	Pr(d)[c]
1	.0	.5	.5	.02
2	.0	.8	.8	.01
3	.0	.0	.5	.20
4	.0	.0	.8	.16

[a] Models correspond to autosomal dominant (models 1 and 2) and recessive (models 3 and 4) patterns of inheritance, with penetrance values of 50% (models 1 and 3) and 80% (models 2 and 4).

[b] A plus sign (+) = wild-type allele at disease locus; d = disease allele at disease locus.

[c] Pr(d) is altered in each model, to maintain a constant prevalence.

risk for first-degree relatives of affected individuals,[2,3,20] and (v) the aggregation of affected individuals in families, instead of a clear Mendelian pattern of inheritance.[2,3] A systematic search for genetic factors underlying this disorder has not been previously undertaken. Additionally, linkage analysis in complex traits is not exempt from challenges, because of the characteristic genetic heterogeneity and the potential presence of numerous susceptibility alleles. These factors may be further complicated, in the case of AA, by the challenges inherent in defining the phenotype, exemplified by a range in ages of onset, the sometimes subtle nature of presenting signs, and the waxing-and-waning nature of the disease.

Until now, genetic studies in AA have been limited to association analyses, which suggest that a permissive human leukocyte antigen (HLA) status may potentiate the development of the disease.[12] In early attempts to identify a genetic component of AA, a number of association studies with candidate genes have been conducted. Association between AA and particular *HLA* alleles (MIM *604305 and MIM *142860), interleukin-1 (*IL1* [MIM *147760 and MIM *147720]) cluster genes, and the myxovirus resistance 1 gene (*MX1* [MIM *147150]), on chromosome 21, have been suggested (for a review, see the work of Green and Sinclair[2] and McDonagh and Tazi-Ahnini[3] and references therein). Significant association has been reported between AA and *DQB1*0301* (for severe AA) and *DRB1*1104* alleles.[12,21] A family-based study has revealed that 85% of patients with AA carry *DQB1*03* alleles, compared with 46% of controls.[22] Those authors also have shown linkage to *HLA-DQB* and *HLA-DR*. Recently, two new positive associations have been described. The MHC class I chain-related gene A (*MICA* [MIM *600169]) has been identified as both a potential candidate gene and a part of an extended *HLA* haplotype that may contribute to the susceptibility to and severity of AA.[23] Additionally, the gene encoding the lymphoid protein tyrosine phosphatase (*PTPN22* [MIM *600716]) has been shown to be associated with severe forms of AA.[24]

The presence of a perifollicular T-cell infiltrate suggests an important role for cytokine production in the pathogenesis of AA. Likewise, it is possible to transfer the disease with lesional human lymphocytes into an SCID mouse grafted with human scalp skin.[25] Along these lines, several authors have suggested association between the more severe forms of AA and the IL-1 receptor antagonist gene (*IL1RN* [MIM *147679]), in particular, allele *IL1RN*2,* and the IL-1 receptor antagonist homologue (*IL1F5* [MIM *605507]).[26–28] Furthermore, there is an increased prevalence of AA among patients with Down syndrome (MIM #190685) (9%) compared with among control individuals (0.1%).[29] The *MX1* gene maps to chromosome 21 and encodes an interferon-inducible protein highly expressed in lesional anagen hair bulbs from patients with AA. Tazi-Ahnini et al.[30] showed significant association between a SNP located within *MX1,* 9,959 bp from the transcription start site, and patchy AA. Finally, an association between autoimmune polyglandular syndrome type 1 (APS1 [MIM #240300]), caused by mutations in the *AIRE* gene (MIM *607358), on chromosome 21, and AA has also been reported,[31] with AA observed in 37% of patients with APS1. Despite the genetic associations described above, it is likely that these alleles account for only part of the genetic susceptibility to AA. It is noteworthy that cosegregation with *HLA* was excluded in two Israeli families.[32]

A second line of evidence for the genetic basis of AA comes from the study of animal models. Particularly, the C3H/HeJ mouse is an inbred laboratory strain that spontaneously develops an adult-onset disease that resembles adult-onset AA in humans. Sundberg et al.[33] have identified four genetic susceptibility loci on mouse chromosomes 8 (*Alaa3*), 9 (*Alaa2*), 15 (*Alaa4*), and 17 (*Alaa1*), wherein Alaa1 corresponds to HLA orthologs.

The lack of knowledge of the etiology of AA, the psychological impact on the quality of life of patients with AA, and the absence of an effective treatment underscore the importance of identifying the mechanisms underlying the disease. With this in mind, we initiated a comprehensive genetic analysis of families with multiple affected individuals, in search of genes that contribute to the development of AA.

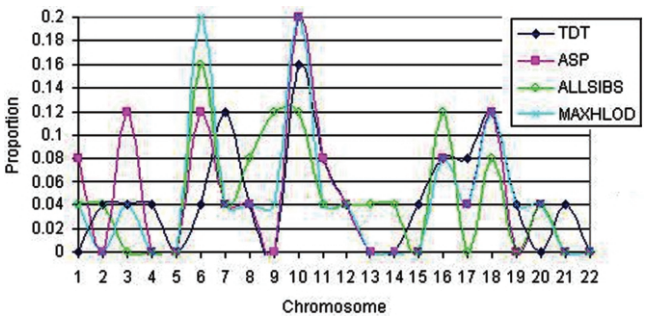

Figure 2. Proportion of top 25 scores on a given chromosome for analysis of pedigrees with AA by use of each of the four test statistics.

Table 2. Top 15 Results for Four Genetic Statistical Methods Applied to the 20 Genotyped Pedigrees with AA

Method and Result Rank	Chromosome	Locus	LOD
MAXHLOD:			
1	6	*D6S1009*	3.554
2	16	*D16S753*	2.007
3	6	*D6S2427*	1.707
4	10	*D10S1239*	1.691
5	2	*D2S1776*	1.545
6	9	*D9S301*	1.518
7	1	*D1S1612*	1.434
8	10	*D10S2481*	1.384
9	17	*D17S1301*	1.228
10	6	*D6S1270*	1.173
11	12	*D12S1064*	1.129
12	9	*D9S930*	1.081
13	6	*D6S1003*	1.068
14	18	*D18S976*	1.055
15	6	*D6S1281*	1.034
ASP:			
1	6	*D6S1009*	4.831
2	18	*D18S535*	2.314
3	6	*D6S1003*	1.660
4	17	*D17S1301*	1.388
5	11	*D11S1390*	1.213
6	17	*D17S1293*	1.126
7	17	*D17S916*	.967
8	16	*D16S3253*	.964
9	6	*D6S1040*	.919
10	16	*D16S403*	.915
11	16	*D16S753*	.899
12	3	*D3S2398*	.881
13	10	*D10S1239*	.844
14	2	*D2S1776*	.820
15	2	*D2S1353*	.874
ALLSIBS:			
1	6	*D6S1009*	2.762
2	10	*D10S2481*	1.354
3	2	*D2S338*	1.340
4	2	*D2S1776*	1.025
5	10	*D10S674*	.850
6	1	*D1S1612*	.845
7	10	*D10S1230*	.836
8	6	*D6S1270*	.809
9	6	*D6S1056*	.775
10	9	*D9S930*	.755
11	10	*D10S1239*	.747
12	8	*D8S1179*	.704
13	11	*ATA34E08*	.696
14	13	*D13S779*	.691
15	9	*D9S301*	.690
TDT:			
1	17	*D17S1301*	1.930
2	9	*D9S1122*	1.659
3	17	*D17S1290*	1.505
4	18	*D18S535*	1.410
5	7	*D7S2846*	1.389
6	1	*D1S1612*	1.383
7	1	*D1S1595*	1.312
8	18	*D18S1116*	1.245
9	18	*D18S976*	1.148
10	9	*D9S910*	1.117
11	18	*D18S59*	1.115
12	6	*D6S1003*	.942
13	5	*D5S1354*	.918
14	16	*D16S686*	.837
15	1	*D1S1597*	.820

Material and Methods

Ascertainment of Families

Families were recruited through a patient who received a diagnosis of AA. The inclusion criteria required that families have two or more affected relatives. With this requirement as a starting point, all family members willing to participate were recruited for the study. Pedigrees were enrolled from the United States, primarily through the National Alopecia Areata Registry, and from Israel. Those family members for whom clinical data were not available were classified as "unknown" for linkage purposes.

Clinical examiners diagnosed AA in the patients before the genetic studies. At the time of consultation, blood samples were drawn and written informed consent was obtained from all participants. The study was approved by the local institutional review boards. Overall, 38 pedigrees, consisting of 102 affected and 118 unaffected or unknown individuals who participated in the study, were collected (examples shown in fig. 1*E*). On average, each family contained three affected individuals. The largest pedigree, HAA01, originating from Israel, consisted of 22 participating family members, 10 of whom received a diagnosis of AA (fig. 1*E*).

Genotyping

Genomic DNA was extracted using the PureGene DNA Isolation Kit (Gentra Systems). Twenty pedigrees (fig. 1*E*) were initially genotyped using a semiautomated high-throughput genotyping approach with fluorescently labeled microsatellite markers.[34–36] A panel of 342 microsatellite markers was used, with an average marker spacing of 10 cM and an average heterozygosity of 0.77. Most of the markers were chosen from version 8.0 of the Marshfield fluorescence-labeled genome screening set. DNA samples and PCR reagents were aliquoted with a TECAN Genesis RSP 150 robotic workstation. Multiplex PCR was performed in 384-well plates (Marsh) in PTC 225 thermocyclers (MJ Research). An average of 50 ng of genomic DNA was amplified in 10-μl PCRs containing 0.15–0.2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 units of *Taq* Platinum polymerase (Invitrogen). The primer concentration was adjusted (1–50 pmol) to achieve even amplification of each marker locus contained in the multiplex PCRs. As described elsewhere,[37] and to improve allele-calling, the last nucleotide of the reverse, nonfluorescent primer was modified to a guanine to promote the nontemplated addition of adenine by *Taq* DNA polymerase onto the complementary, fluorescence-labeled strand. DNA from CEPH control individuals was used as a size standard for every marker locus. PCR products were electrophoresed on 377 DNA sequencers (PE Applied Biosystems). Raw data from the PCR products were collected by PRISM 377XL data-collection software (PE Applied Biosystems), and the products were sized by GENESCAN version 2.1 and GENOTYPER v.1.1.1. The genotypes were imported to LABMAN[38] for allele binning, Mendelian checking, and generation of linkage files. The PEDCHECK program[39] was used to check for genotype errors. The markers that showed genotype errors were recoded to unknown genotypes in each family in which a genotype error was observed. For all multipoint runs, the MEGA2 program[40] was used to format pedigree data for computation of the likelihood-ratio Z (Z_{lr}) statistics.

As follow-up to the genome scan, fine mapping with microsatellite markers was performed by deCODE Genetics for the entire cohort of 38 families. For each marker, the forward primer was fluorescently labeled. The primer pairs were extensively tested

for optimizing the multiplex PCR reactions for cost benefits. PCR amplifications were set up on Zymark ALH 400, were run on MJR Tetrad, and were pooled on Gilson Cyberlab C200 robots. The reaction volume was 5 μl, and, for each PCR, 20 ng of genomic DNA was amplified in the presence of 2 pmol of each primer, 0.25 U AmpliTaq Gold, 0.2 mM dNTPs, and 2.5 mM $MgCl_2$ (buffer was supplied by the manufacturer, Applera). Cycling conditions were as follows: at 95°C for 10 min, followed by 37 cycles at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR products were supplemented with the internal size standard GS500-LIZ, and the pools were separated and detected on 3730 Sequencers. Alleles were automatically called using DAC, an allele-calling program developed at deCODE Genetics,[41] and the program deCODE GT was used to fractionate called genotypes, according to quality, and to edit, when necessary.[42]

Six chromosomal regions on four chromosomes (6, 10, 16, and 18) were genotyped, corresponding to the six intervals: (i) the *HLA* region on chromosome 6, analyzed using a high-density panel of 60 microsatellite marker loci at an average marker spacing of 0.09 cM, which spanned the interval flanked by *D6S2219* and *D6S1560* (2.37 cM); (ii) chromosome 6 interval *D6S1040–D6S1003* (16.72 cM; 12 marker loci); (iii) chromosome 10 interval *D10S674–D10S2481* (10.26 cM; 11 marker loci); (iv) chromosome 10 interval *D10S1239–D10S1230* (23.22 cM; 17 marker loci); (v) chromosome 16 interval *D16S403–D16S3098* (60.13 cM; 34 marker loci); and (vi) chromosome 18 interval *D18S59–D18S1157* (61.03 cM; 29 marker loci). Marker locations are reported in Haldane map units.

Statistical Analysis

Initial genome scan.—A two-step linkage analysis was performed on the collection of 38 pedigrees (102 affected and 118 unaffected participating individuals). In the first stage, a genomewide scan was conducted for a collection of 20 pedigrees and a total of 131 DNA samples (69 affected and 62 unaffected family members; fig. 1*E*). The test statistics applied to the data set obtained in the genomewide scan were (i) the heterogeneity LOD score,[43,44] maximized over four settings of penetrance parameters (hereafter, "MAXHLOD") (see table 1 for the penetrance settings used); (ii) the mean test for affected sib pairs, as implemented in the ANALYZE program[45] (hereafter, "ASP"); (iii) a test of allele sharing that uses all sibs[45] (hereafter, "ALLSIBS"); and a likelihood version of the transmission/disequilibrium test,[46] as developed by Terwilliger[47] (TDT-like, hereafter referred to as "TDT").

Table 3. Summary of Fine-Mapping Results for U.S., Israeli, and Combined Pedigrees

Chromosome and Sample	Maximum LOD	Position (cM)	Method	Marker
6:				
U.S.	3.03	138.78	ASP	*D6S270*
Israeli	1.42	142.71	ASP	*D6S1009*
Combined	2.88	142.71	ASP	*D6S1009*
10:				
U.S.	1.88	41.4	TDT	*D10S1661*
Israeli	1.63	51.42	HRR	*D10S2481*
Combined	1.63	51.42	HRR	*D10S2481*
16:				
U.S.	1.28	69.14	ASP	*D16S415*
Israeli	2.14	65.35	ASP	*D16S2623*
Combined	3.11	69.14	ASP	*D16S415*
18:				
U.S.	3.73	21.74	Z_{lr}	*D18S967*
Israeli	1.38	26.03	HRR	*D18S1163*
Combined	3.93	21.74	Z_{lr}	*D18S967*

For the ASP test, when there are multiple sibs in a sibship, ANALYZE weights the sib pairs according to the sibship size, as follows two sibs equal one sib pair, three sibs equal two sib pairs, and four sibs equal three sib pairs. As noted by Terwilliger in the user notes for ANALYZE, "This weighting has been selected to conform with the information content of phase-unknown nuclear pedigrees in linkage analysis."[48(p9)] A detailed discussion has been published.[49,50]

Our rationale for the use of these different tests was described elsewhere.[51] Briefly, for the MAXHLOD calculations, we applied a model-based linkage analysis in which the LOD score was calculated under both autosomal dominant and autosomal recessive patterns of inheritance. For both models, two different values of penetrance were considered. ASP, ALLSIBS, and TDT tests were chosen because they are all genetic-model free,[52,53] in the sense that they do not require a specification of the genetic model parameters (penetrance and disease-allele frequency). MAXHLOD was chosen because it has been shown that it has at least as much power to localize disease loci as do ASP and ALLSIBS,[54] and, under

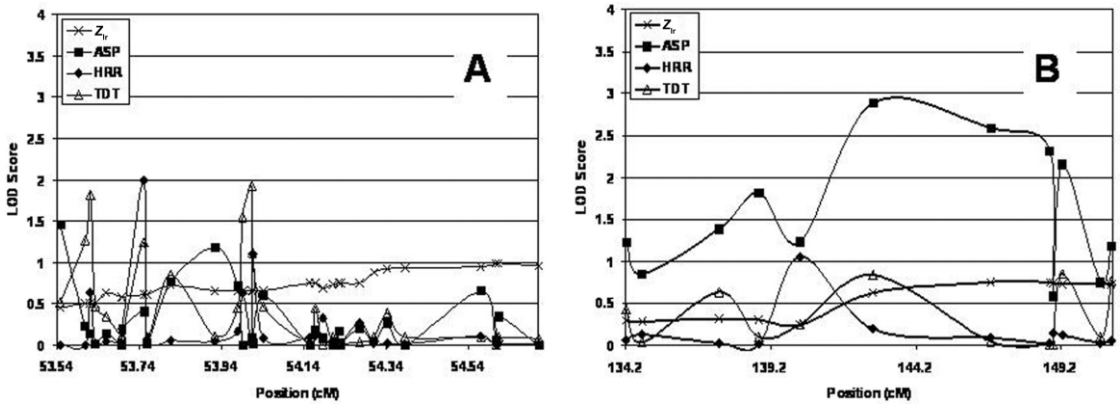

Figure 3. Results of the fine-mapping study for chromosome 6. *A,* Region 52.33–54.71 cM. *B,* Region 134.2–150.93 cM.

certain circumstances, it is a more precise indicator of the location of a disease locus than are statistics such as ASP and ALLSIBS.[55] Finally, the TDT was chosen because it has been shown that it may be more powerful than linkage tests (e.g., MAXHLOD, ASP, and ALLSIBS) when linkage and linkage disequilibrium exist between a disease and a marker locus.

All results are presented as LOD scores. The TDT *P* value was converted to a LOD score by use of the following approximation. Let *P* be the *P* value for the TDT statistic. Because TDT is asymptotically distributed as χ^2 with 1 df, we convert *P* to a LOD score via the formula $\chi^{-1}(P)/4.6$, where χ^{-1} is the inverse of the one-tailed probability of the χ^2 distribution. An identical conversion was used for the haplotype-based haplotype relative risk (HRR) minus 2 times *n* statistic (HRR − 2 × *n*; see the "Fine-mapping analyses" section).

Fine-mapping analyses.—For fine-mapping purposes, we focused on the marker loci that showed significant test scores (the top 10% of scores observed across all marker loci) for at least two of the test statistics listed above. At this stage, we were more concerned with power than with inflation of the false-positive rate.[56]

We performed fine-mapping analyses, using two-point and multipoint genetic model–free statistics. The two-point method was the ASP test. The multipoint method was the Kong and Cox[57] Z_{lr} statistic, which is implemented in the GENEHUNTER-PLUS software. Although we used affected and unaffected individuals in our initial genome scan (for the MAXHLOD and ALL-SIB statistics), we decided to focus on "affecteds-only" statistics in the fine-mapping analyses. Our reasoning was that the ASP method was generally more powerful (i.e., yielded higher LOD scores) than were the MAXHLOD and ALL-SIB statistics. We hypothesized that decrease in power for methods using unaffected individuals may stem from the fact that they are truly affected but did not present with symptoms at the time of diagnosis. The TDT statistic is also an affecteds-only statistic that takes advantage of any linkage disequilibrium that may occur between marker and disease locus. We added a family-based test of association, the HRR.[58] The HRR statistic tests for association in the presence of linkage—unlike the TDT, which tests for linkage in the presence of association.[59] More intuitively, the HRR tests whether the risk alleles are a single allele or a small set of alleles, whereas the TDT tests whether recombination between the trait and marker loci is small (close to 0). Our rationale for using a family-based test of association was based on the observation that, for several autoimmune diseases, associations with the MHC have been described, despite weak or no evidence of linkage. This phenomenon is thought to occur because of the high frequency of the associated allele, which creates multiple MHC haplotypes among affected members within families.

We use the HRR − 2 × *n* version of the HRR, as implemented in the ANALYZE program. As Terwilliger notes in the README notes on the program, "The test performed is that standard 2 × *n* table χ^2 test, which has *n* − 1 df (where there are *n* alleles whose frequencies are compared in case and control samples). This test is less powerful by far when there is only one associated allele (i.e., linkage disequilibrium from a founder effect), but can be more sensitive when there are higher order associations with different alleles."[48(p7)] Particularly for the MHC region, we expect that the latter situation will be true. The case and control samples for family-based data refer to the transmission of a particular allele to an affected child (case sample) and the nontransmission of a particular allele to an affected child (control sample).

Results

Initial Genome Scan

The top 15 scores resulting from each of the four statistical methods used are presented in table 2. A total of 10 marker loci resulted in LOD scores >2 (5 are shown in table 2). Among them, marker *D6S1009,* at 6q23.3, reached the highest linkage signal, with a MAXHLOD score of 3.554 and an ASP LOD score of 4.831. Even after correction for multiple testing, both scores are significant at the .05 level genomewide.[60,61] Moreover, this locus showed the top score for three of the four statistical methods considered (table 2). It is interesting to note that several marker loci appear in the list of top scores for a few of the statistical methods. Although some of the methods may have correlated results,[62] they are not identical. Finally, marker loci *D6S1281* and *D6S2427,* which are located in the vicinity of the *HLA* region on chromosome 6, were among the markers with the top 15 MAXHLOD scores ($Z_{\max}$ = 1.034 and 1.707, respectively).

Because certain chromosomes appear among the top scores for at least three of the four statistical methods applied (table 2), and given that these statistics test different hypotheses, these results point to regions on chromosomes 1, 2, 6, 9, 10, 16, 17, and 18 as potential loci harboring AA-susceptibility genes.

As an exploratory tool that might indicate which chromosomes harbor susceptibility loci for AA, in figure 2, we plotted the proportion of the top 25 scores for each test statistic that appear on a given chromosome. Importantly, for four chromosomes (6, 10, 16, and 18), at least three of the four test statistics have at least 8% of their top 25 scores on that chromosome. The 8% value is 4% higher than the value expected by random selection. Furthermore, if we consider a proportion of ⩾0.12, then, for three chromosomes (6, 10, and 18), three of the four tests have at least 12% of their top 25 scores on that chromosome. Thus, the results of the initial genome scan indicate several suggestive regions of linkage; the most significant LOD scores were 4.8 on chromosome 6 at marker *D6S1009* and 2.3 (for a marker other than *D6S1009*) on chromosome 18 at marker *D18S535*.

We performed multipoint linkage analysis, using the MAXHLOD statistic with the initial genome scan data; however, none of the multipoint results were as significant as the two-point MAXHLOD scores. We postulate that this result stems from the fact that the marker density (10 cM) was insufficient to benefit from multipoint linkage analysis (full data not shown).

Fine Mapping

Several authors have defined criteria for declaring suggestive and significant linkage for complex traits, but there is still controversy about what thresholds should be applied and how to extend the theoretical situations on which they are based to real data sets.[63–65] Considering this, we applied the following criteria to the results obtained

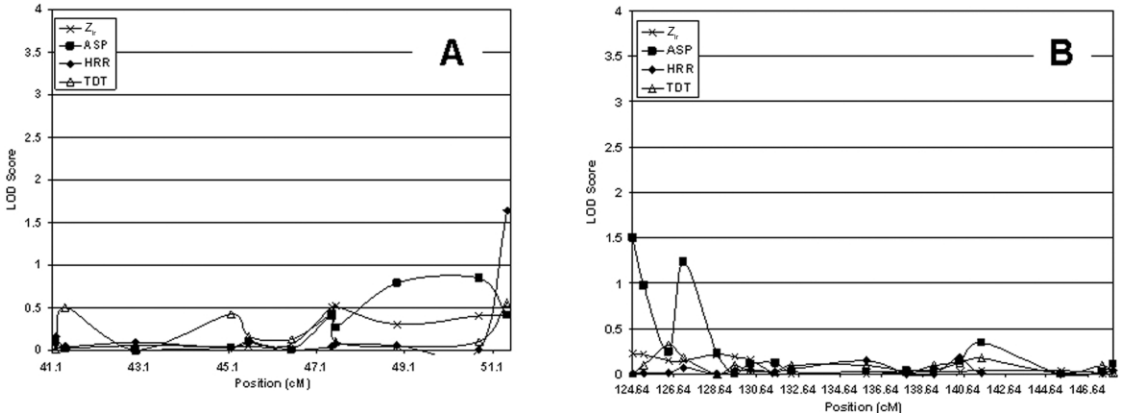

Figure 4. Results of the fine-mapping study for chromosome 10. *A,* Region 41.19–51.42 cM. *B,* Region 124.64–147.86 cM.

in the genome scan, to prioritize the follow-up regions for the second stage of our study: (i) regions yielding LOD scores among the top 15 scores for two or more statistical tests, (ii) marker loci showing LOD scores values >2; and (iii) chromosomal regions "overrepresented" by having consecutive or nearly consecutive marker loci among the top 15 scores for different statistical tests. On the basis of this algorithm, and to optimize the use of DNA samples, we chose to first genotype additional marker loci in six regions on chromosomes 6, 10, 16, and 18 (see the "Material and Methods" section for details on the intervals). The *HLA* region was included in this second stage on the basis of the reports of genetic association of AA with different *HLA* alleles. Regions on chromosomes 1, 2, 9, and 17 will be analyzed in subsequent studies.

The follow-up marker density was increased from 1 marker every 10 cM (in the genome scan) to 1 every 1.82 cM, on average. For the analysis of the *HLA* region, we used a high-density panel of microsatellites designed by deCODE Genetics with marker loci spaced at an average distance of 0.09 cM (see the "Material and Methods" section). Results obtained after genotyping additional microsatellite marker loci are plotted in figures 3–6.

Chromosome 6

On chromosome 6, we fine mapped two regions: 52.33–54.71 cM and 134.2–150.93 cM. In figure 3*A* and 3*B,* we plotted LOD scores for both regions. The largest ASP LOD score observed on chromosome 6 in the fine-mapping data occurred at position 142.7 cM (marker *D6S1009*). The ASP LOD score at that marker is 2.89. This marker locus also showed the most significant ASP LOD score in the original genome scan (table 2). The decrease in significance is because of the addition of new families in the fine mapping, some of which were unlinked to this locus. It is important to note that the next most significant ASP LOD scores all occur for marker loci within ~6 cM of *D6S1009* (fig. 3*B*). For example, the second-most significant ASP LOD score is 2.59, for position 146.8 cM (marker *D6S1569*), and the third-most significant ASP LOD score is 2.31, for position 148.8 cM (marker *D6S1684*).

The most significant TDT LOD score of 1.9 ($P = .0015$) occurred at position 54.01 cM (marker *D6S2889*). As with the ASP LOD scores, the next five most significant TDT LOD scores all occurred within 0.5 cM of this position (fig. 3*A*). Finally, the most significant Z_{lr} LOD score of 1.49 ($P = .004$) occurred at position 54.6 cM (marker *D6S2727*), within 0.6 cM of the most significant TDT LOD score. Another important observation is that the Z_{lr} multipoint LOD scores are almost always more significant than are the ASP two-point LOD scores in the region from 52 cM to 56 cM, whereas the converse is true in the region from 134.2 cM to 150.93 cM (fig. 3*A* and 3*B*).

Consideration of the HRR statistic for these data provided additional information. In particular, marker *D6S273* (position 53.75 cM) showed an HRR LOD score of 1.99 ($P = .001$). This score was the second largest

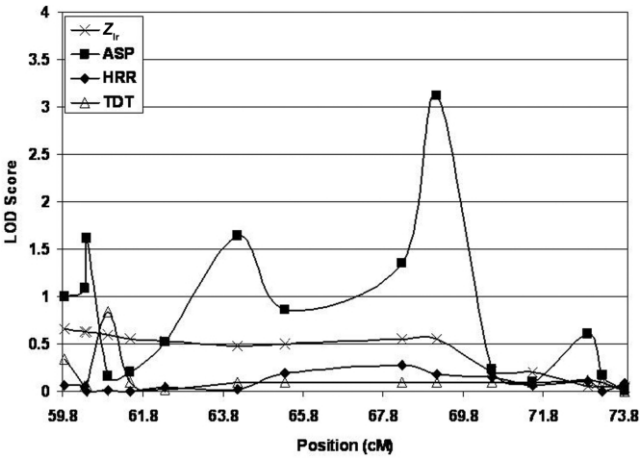

Figure 5. Results of the fine-mapping study for chromosome 16

among HRR LOD scores for all fine-mapping data. It is interesting to note that this marker is in the MHC locus, for which previous associations with AA have been reported.[19,22,23,30,66]

Chromosome 10

On chromosome 10, we fine mapped two regions: 41.19–51.42 cM and 124.64–147.86 cM. In figure 4*A* and 4*B*, we plotted the LOD scores for the three statistics for these two regions. Overall, the highest LOD scores occurred for three marker loci by use of the ASP statistic. Marker *D10S1239*, at position 124.64 cM, showed an ASP LOD score of 1.50 ($P = .004$). Marker *D10S254*, at position 127.08 cM, showed an ASP LOD score of 1.23 ($P = .009$). Finally, marker *D10S1738*, at position 125.15 cM, showed an ASP LOD score of 0.975 ($P = .017$). The largest Z_{lr} LOD score (0.093; $P = .064$) occurred at position 47.53 cM (marker *D10S1734*). The largest LOD score for the TDT method was at position 51.42 cM (marker *D10S2481*). The largest HRR LOD at position 51.42 cM (marker *D10S2481*) was 1.63 ($P = .003$). This score was the third largest among HRR LOD scores for all fine-mapping data.

Chromosome 16

On chromosome 16, we fine mapped the region from 47 cM to 73.82 cM. The most significant result occurred for marker *D16S415*, at position 69.14 cM, where we observed an ASP LOD score of 3.11. The multipoint Z_{lr} LOD score for the same marker was 0.08, indicating that the two-point and multipoint results show substantially different evidence of linkage. The TDT and HRR LOD scores were generally nonsignficant ($P > .10$), with the exception of that for marker *D16S261* (60.925 cM), which displayed a TDT LOD score of 0.84 ($P = .02$).

Chromosome 18

For chromosome 18, we fine mapped the region from 1 cM to 62 cM. The most significant result occurred for marker *D18S967*, at position 21.74 cM, where we observed an Z_{lr} LOD of 3.93 (fig. 6). Relative peaks for the ASP, TDT, and HRR methods also occurred near this position (fig. 6). In fact, the Z_{lr}, TDT, and HRR LOD scores for marker *D18S976* (position 16.55 cM) are in the top 10% of the distribution of LOD scores for each statistic's distribution of fine-mapping LOD scores. It is important to note that, for chromosome 18, the multipoint LOD scores (Z_{lr} values) increased over the two-point ASP LODs in the region from ~10 cM to 30 cM.

Stratified Fine-Mapping Analyses

In table 3, we present results of our stratified fine-mapping analyses for only U.S. pedigrees, only Israeli pedigrees, and all pedigrees combined. As has been shown in other linkage studies,[67–70] stratification of data by a certain criteria (e.g., disease diagnosis or ethnic origin) often will increase

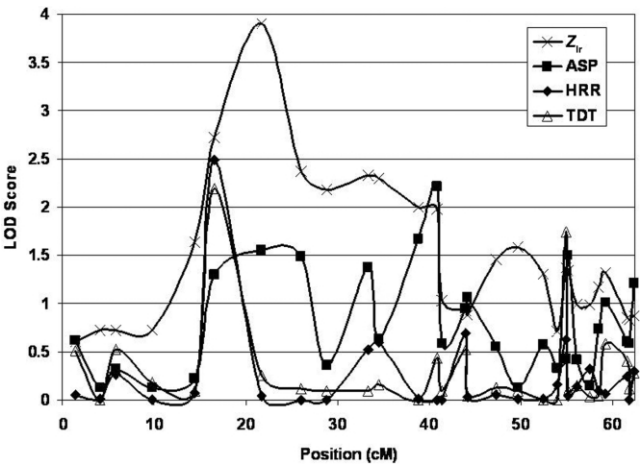

Figure 6. Results of the fine-mapping study for chromosome 18

linkage information in at least one of the substrata. The results for all pedigrees combined are included in table 3 for comparison.

The combined results for chromosome 6 are relatively consistent across all sets of pedigrees. That is, the position of the maximum LOD score is between 138.78 cM and 142.71 cM, and the maximum LOD score was achieved using the ASP method. The maximum LOD score increases to 3.03 when only the U.S. pedigrees are considered, compared with the maximum LOD of 2.88 for all pedigrees (see also fig. 3*B*).

We observed similar results for the chromosome 10 analyses. The maximum LOD score increases slightly from 1.63 for the combined set of pedigrees to 1.88 for only the U.S. pedigrees, and the position of the maximum LOD shifts from 51.42 cM (combined set) to 41.40 cM (U.S. pedigrees).

On chromosome 16, the results for the stratified sets (U.S. and Israeli pedigrees) appear additive. That is, the combined maximum LOD score of 3.11 is approximately the sum of the maximum LOD score for the U.S. set (1.28) and the maximum LOD score for Israeli set (2.14), although the position for the maximum LOD differs by ~4 cM for the U.S. and Israeli sets.

Finally, for chromosome 18, results of the stratification suggest that the maximum LOD score of 3.93 for the combined set of pedigrees is largely from the U.S. pedigrees. The maximum LOD for the only the U.S. pedigrees is 3.73 at the same position (21.74 cM).

Conclusions from the Fine-Mapping Results

Taken together, the fine-mapping results indicated the strongest evidence of linkage on chromosome 18 in the region near 21 cM. It is worth noting that the fine-mapping analyses of chromosome 18 with additional families produced significantly larger LOD scores than those from

the original genome scan, and the maximum multipoint LOD score of 3.9 is highly significant.

The next strongest evidence of linkage occurred for chromosome 6 in the HLA region. We see multiple marker loci with TDT and/or HRR LOD scores >1.5. These results are not surprising, given that population-based association methods have implicated HLA marker loci as being associated with AA. Although there are some two-point (ASP) LOD scores >3 for marker loci on chromosome 16, when the multipoint (Z_{lr}) method is applied, these LOD scores decrease substantially.

Stratification of pedigrees by ethnic origin resulted in an increased linkage signal on chromosomes 6 and 10 for the U.S. pedigrees, no increase in the linkage signal on chromosome 16, and the observation that the maximum LOD score for chromosome 18 appears to be from the U.S. pedigrees. As a methodologic note, the TDT and HRR methods provided highly correlated results, and, in fact, the correlation among the TDT and HRR *P* values (−log transformed) was 0.32 ($P < .001$ for test of correlation).

Discussion

The mapping of complex disorders has only recently begun to yield successes in gene identification. The identification of susceptibility genes for psoriasis and atopic dermatitis are among the most significant results obtained to date for complex diseases in dermatology.[71–76] One of the first significant findings in complex-disease mapping was the identification of alleles predisposing to Crohn disease (MIM #266600) in the *NOD2* gene (MIM *605956).[77,78] More recently, an allele of the complement factor H gene (*CFH* [MIM *134370]) was significantly associated with age-related macular degeneration (MIM #153800).[79–82] In all examples, the genetic location of the disease susceptibility locus was first identified by performing genome scans on families.[71,75,83] Subsequent to the linkage analysis, association studies were used to determine the particular alleles that confer disease susceptibility. Although many studies of complex diseases have focused on the collection of individual cases or sib-pair samples because of the difficulty of finding a significant number of pedigrees segregating the disease, these types of studies are more vulnerable to the effects of genetic heterogeneity and the polygenic nature of such diseases. Therefore, the identification of novel disease loci has traditionally been accomplished with family-based linkage studies. For this reason, we chose to approach this first genomewide susceptibility search of AA by using a collection of pedigrees with multiple affected individuals. Furthermore, 14 of the pedigrees originate from Israel, increasing the probability that they share underlying genetic factors.

The statistical tests used in this study were chosen because they all are genetic model-free tests,[52,53] in the sense that (with the exception of MAXHLOD) they do not require a specification of the genetic-model parameters (penetrance and disease-allele frequency). MAXHLOD is a parametric linkage analysis in which the LOD score is calculated under autosomal dominant and recessive patterns of inheritance and penetrance values of 50% and 80% (table 1). It has been shown that it is at least as powerful in localizing disease loci as tests like ASP and ALLSIBS[54] and is, under certain circumstances, a more precise indicator of the location of a disease locus than are statistics like ASP and ALLSIBS.[55] Several authors have used parametric linkage analyses to look for susceptibility loci contributing to complex traits, since, when calculated under both dominant and recessive models even though they do not describe the pattern of inheritance of such diseases, these methods can be a powerful way to localize susceptibility loci.[84] Furthermore, these methods can be useful in a sample like ours, where many of the affected pairs are not siblings.

TDT was chosen because it has been shown that it may be more powerful than linkage tests (MAXHLOD, ASP, ALLSIBS), when there is linkage and linkage disequilibrium between a disease and marker locus. Some of the families with AA collected in Israel are of Ashkenazi and Sephardic Jewish extraction. These are considered genetically isolated populations, and the extent of linkage disequilibrium is therefore thought to extend over larger regions of the human genome.[85,86] Overall, the strategy used to identify chromosomes of interest for follow-up is similar to exploratory methods employed by other researchers analyzing genome-scan data for complex traits.[87] Previous authors subdivided the genome into chromosomal bins, whereas, in this work, we examine whole chromosomes at a time. We consider the whole chromosome as the unit of measure because it has been observed, in simulated data sets, that the methods we employ have better power to determine the correct chromosome than to determine a particular subregion of a chromosome harboring a disease-susceptibility locus.[56]

In this study, we did not specifically model sporadic cases, where the probability of being affected given two copies of the wild-type allele at the disease locus, Pr(aff/++), could carry one or two copies of mutated allele; that is, Pr(aff/++) is perhaps nonzero. However, our previous research[55] suggests that the effect of sporadic cases can be modeled by locus heterogeneity. Therefore, we used the MAXHLOD statistic rather than the MAXLOD statistic.

It has been suspected for many years that an autoimmune pathogenesis underlies AA, yet firm evidence of an autoantigen is lacking.[11–16] The results of association studies with *HLA* in AA suggest a role,[12] although perhaps it explains only a part of the genetic susceptibility to AA. When compared with the four AA-susceptibility loci identified in the C3H-HeJ mouse model,[33] our results overlap at the *HLA* locus. In our data sets, the two marker loci in the vicinity of the *HLA* locus yield MAXHLOD values of 1.034 (*D6S1281*) and 1.707 (*D6S2427*). The other three loci in the mouse (on mouse chromosomes 8, 9, and 15) correspond to regions on 10 different human chromosomes (4, 5, 8, 11, 12, 13, 16, 17, 19, and 22). Although

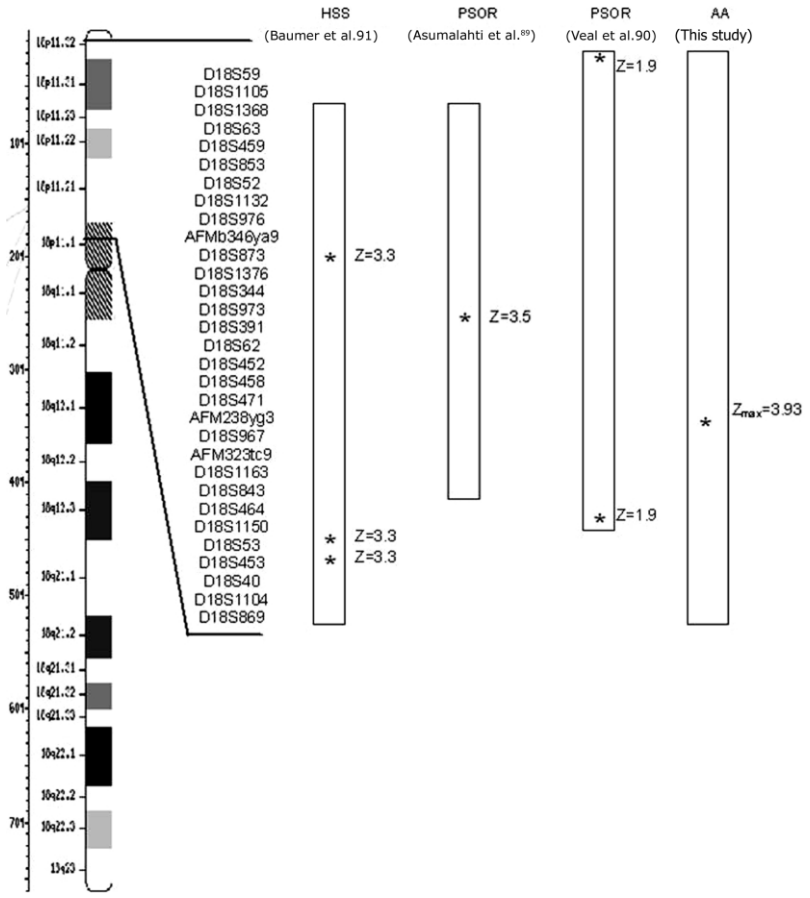

Figure 7. Comparison of chromosome 18 results with published studies.[90,91] Boxes represent regions that showed linkage. Asterisks (*) indicate marker loci with the highest linkage scores. HSS = hereditary hypotrichosis simplex; PSOR = psoriasis.

the majority of these loci do not correspond to our findings, the region on chromosome 8 does correspond to part of the region that we fine mapped on human chromosome 16. Since this study represents a first attempt to identify genetic factors contributing to AA in humans, our results should be replicated in an independent population. It is possible that new loci will be identified once the sample is extended and further suggestive chromosomal regions are tested, such as those on chromosomes 1, 2, 9, and 17.

Several studies published elsewhere have reported the association of AA with various genes, including *MX1* and *AIRE* on chromosome 21 and *PTPN22* on chromosome 1. None of these regions were implicated in our study.

We noted two significant peaks of linkage on chromosome 6, one on each arm. The main peak was on 6q (ASP LOD score 2.89), outside the HLA region. The HLA region on 6p also showed suggestive linkage (TDT and HRR LOD score of 1.9), which is consistent with previous reports.[19,22,23,30,66]

Two of the regions identified in this study have previously been implicated in conferring susceptibility to psoriasis. The region on chromosome 16 was identified in a genome scan performed on patients with psoriasis ($Z_{max} = 2.31$)[88] and also overlaps with a region near a Crohn disease–susceptibility locus.[83] On chromosome 18, we observed consistent linkage peaks >3.0 ($Z_{max} = 3.93$). This same region of chromosome 18p also contains a psoriasis-susceptibility region that was identified independently in families from the United Kingdom ($Z = 1.97$) and Finland ($Z = 3.58$).[89,90] There has also been a reported association between deletion of chromosome 18p and psoriasis vulgaris (fig. 7).[66] Interestingly, this same area of chromosome 18 has also been implicated by linkage in autosomal dominant hereditary hypotrichosis simplex ($Z = 3.31$), although no causative gene has been identified yet.[91] Taken together, these lines of evidence suggest that a gene(s) on chromosome 18p is linked to AA and may also be involved in other inherited skin and hair disorders.

Understanding the genetic factors underlying AA may help to elucidate the mechanisms responsible for the disease, as well as to define at-risk individuals, to improve the disease prognosis, and to determine its response to environmental triggers; eventually, it could lead to the design of new treatment strategies. Initially, the genes in AA could provide targets for transgenic and knockout mice, which would be essential for in vivo testing of new

therapies. Ultimately, it is anticipated that discovery and modulation of the genes in AA will provide novel therapeutic targets for this psychologically devastating disorder.

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Web Resources

The URLs for data presented herein are as follows:

National Alopecia Areata Foundation, http://www.naaf.org/
National Alopecia Areata Registry, http://www.mdanderson.org/departments/alopecia/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AA, *HLA* alleles, *IL1, MX1, MICA, PTPN22, IL1RN, IL1F5,* Down syndrome, ASP1, *AIRE,* Crohn disease, *NOD2, CFH,* and age-related macular degeneration)

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