ORIGINAL ARTICLE

NALP1 in Vitiligo-Associated Multiple Autoimmune Disease

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ABSTRACT

BACKGROUND

Autoimmune and autoinflammatory diseases involve interactions between genetic risk factors and environmental triggers. We searched for a gene on chromosome 17p13 that contributes to a group of epidemiologically associated autoimmune and autoinflammatory diseases. The group includes various combinations of generalized vitiligo, autoimmune thyroid disease, latent autoimmune diabetes in adults, rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease.

METHODS

We tested 177 single-nucleotide polymorphisms (SNPs) spanning the 17p13 linkage peak for association with disease and identified a strong candidate gene. We then sequenced DNA in and around the gene to identify additional SNPs. We carried out a second round of tests of association using some of these additional SNPs, thus elucidating the association with disease in the gene and its extended promoter region in fine detail.

RESULTS

Association analyses resulted in our identifying as a candidate gene *NALP1*, which encodes NACHT leucine-rich-repeat protein 1, a regulator of the innate immune system. Fine-scale association mapping with the use of DNA from affected families and additional SNPs in and around *NALP1* showed an association of specific variants with vitiligo alone, with an extended autoimmune and autoinflammatory disease phenotype, or with both. Conditional logistic-regression analysis of *NALP1* SNPs indicated that at least two variants contribute independently to the risk of disease.

CONCLUSIONS

DNA sequence variants in the *NALP1* region are associated with the risk of several epidemiologically associated autoimmune and autoinflammatory diseases, implicating the innate immune system in the pathogenesis of these disorders.

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Autoimmune and autoinflammatory diseases are a group of about 80 disorders that can involve almost any tissue, organ, or system.[1] A major source of illness and death, these diseases together affect 15 to 25 million people in the United States,[2] particularly women,[3,4] in whom they rank among the top 10 causes of death.[5] The risk of autoimmune and autoinflammatory diseases is thought to depend on interactions between environmental factors and specific variants of specific genes, some of which may confer a risk that an individual disease will develop, and others a risk that several different diseases will develop.[6]

Indeed, many patients eventually have more than one autoimmune or autoinflammatory disease. Various names have been applied to different combinations of so-called multiple autoimmune disease, such as Schmidt's syndrome and autoimmune polyglandular syndromes. A few very rare multiple autoimmune disease syndromes result from mutations in single genes[7]; however, most cases of multiple autoimmune disease do not follow mendelian patterns of inheritance, but rather have complex inheritance patterns. Susceptibility genes in these cases probably fall into two categories: some may specifically predispose patients to one or more of the component diseases, whereas others may affect the susceptibility of patients to autoimmune and autoinflammatory disease in general. The latter type of gene may represent a target in the treatment or even prevention of several different diseases.

We[8,9] and others have observed that, among patients with generalized vitiligo, there is an increased frequency of several other autoimmune and autoinflammatory diseases, particularly autoimmune thyroid disease (Graves' disease and autoimmune hypothyroidism), latent autoimmune diabetes in adults, rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease. There is also an increased frequency of these same disorders among first-degree relatives of patients with vitiligo, suggesting that some families have a genetic predisposition to this group of autoimmune and autoinflammatory diseases. By testing for genetic linkage between disease and polymorphic DNA markers spanning the whole genome in families with vitiligo and other autoimmune and autoinflammatory diseases, we have identified several chromosomal regions (or loci) that appear to contribute to this epidemiologic association, including one on chromosome 17p13.[10] This genomic region also appears to contribute to systemic lupus erythematosus in members of families who inherit lupus together with either vitiligo[11] or various other autoimmune and autoinflammatory diseases.[12] This finding suggests that chromosome 17p13 is involved in the susceptibility to multiple autoimmune disease. To identify the autoimmunity susceptibility gene in the 17p13 region, we performed fine-scale genetic association and DNA sequence analyses in 114 families with vitiligo and associated autoimmune and autoinflammatory diseases.

METHODS

SUBJECTS

We obtained DNA samples from 656 persons from 114 extended families with multiple autoimmune disease associated with vitiligo from the United States and United Kingdom between 1996 and 2005. All families were white (as self-reported on multiple-answer questionnaires; see the Supplementary Appendix, available with the full text of this article at www.nejm.org) and were selected on the basis of having two or more family members with generalized vitiligo (multiplex families) and at least one having one or more of the other autoimmune and autoinflammatory diseases epidemiologically associated with vitiligo (autoimmune thyroid disease, rheumatoid arthritis, latent autoimmune diabetes in adults, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease).[8,9] We studied two series of families. The first series comprised the 51 extended families (333 persons) we previously studied to map a vitiligo–multiple autoimmune disease locus to 17p13,[10] and the second series comprised 63 similar, independent families (323 persons). Clinical information on all families from the first series and on about half the families from the second have been reported previously.[9]

Available affected and unaffected family members completed a detailed questionnaire to provide the clinical history with regard to approximately 50 autoimmune and autoinflammatory diseases and immune-related diseases, including vitiligo, autoimmune thyroid disease (Graves' disease and autoimmune hypothyroidism), rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, Addison's disease, and

diabetes (for which type, age of onset, and course of treatment were specified). All patients with vitiligo provided a skin-lesion map.

The study investigators reviewed all data from the questionnaires and the lesion maps and the study staff examined most family members, both affected and unaffected. Inclusion criteria for family members with generalized vitiligo were the presence of depigmented patches of skin that were acquired; that had changed in extent and boundaries over time; that were nonfocal and bilateral; that typically initially involved the fingers, hands, feet, face, or crural areas; and that were not associated with concurrent underlying eczema or psoriasis or with exposure to depigmenting chemicals. Family members with diagnoses that were considered to be questionable on the basis of standard diagnostic criteria[13] were excluded. Table 1 provides a summary of autoimmune diseases in the 114 study families (see Table 1 in the Supplementary Appendix for details of the clinical diagnoses).

Our study was approved by the Colorado Multiple Institutional Review Board and the South Thames Regional Multicentre Research Ethics Committee. Written informed consent was obtained from all participants.

GENOTYPING

DNA was prepared from peripheral-blood specimens with the use of a genomic DNA purification kit (Puregene, Gentra Systems) or from saliva specimens with the use of a DNA self-collection kit (Oragene, DNA Genotek). We initially used the Illumina genotyping service to genotype family members in the first series, assaying 177 known single-nucleotide polymorphisms (SNPs) selected from the Illumina SNP Knowledge Resource. Each SNP had a minor allele frequency exceeding 0.10 (i.e., the less common variant of the SNP occurred on at least 10% of chromosomes) in whites; altogether, these SNPs captured approximately 18% of the common genetic variation ($r^2 \geq 0.5$) across the genetic-linkage region of chromosome 17p (approximately 11.3 cM, or 6.19 Mb). We genotyped family members in the second series for the 23 SNPs that were significantly associated with disease in the first series according to both the pedigree disequilibrium test[14] and the family-based association test.[15] We then genotyped two insertion–deletion polymorphisms and 78 additional SNPs in all 114 families (both series combined). We identified most of these 78 SNPs by sequencing *NALP1*, the gene for NACHT leucine-rich-repeat protein 1, and its extended promoter region in 15 genetically informative family members. Additional details of genotyping are provided in the Supplementary Appendix.

Table 1. Autoimmune and Autoinflammatory Disease Phenotypes in 114 Multiplex Families.*

Disease Phenotype	No. of Cases
Vitiligo only	219
Vitiligo and autoimmune thyroid disease	70
Vitiligo, autoimmune thyroid disease, and other, nonthyroid autoimmune disease	20
Vitiligo and other autoimmune disease	60
Autoimmune thyroid disease only	86
Autoimmune thyroid disease and other autoimmune disease	23
Other autoimmune disease only	89

* A total of 567 family members reported at least one autoimmune and autoinflammatory disease; 175 reported more than one disease.

DNA SEQUENCING

Having narrowed the 17p13 autoimmunity locus to *NALP1* and its extended promoter region, we sought to identify DNA sequence variants within this region that we could use for further tests of association and for identification of the causal SNP or SNPs. We therefore sequenced 82.9 kb of the *NALP1* gene and its extended promoter region in each of four parents (two from each series) who were heterozygous for a haplotype (a set of SNP alleles that occur on a single chromosome) of three adjacent Illumina SNPs (rs3926687, rs2733359, and rs878329) that initially appeared to confer a high risk (haplotype 1) and who had transmitted this haplotype to at least one affected offspring. We sequenced the same region in 11 unrelated patients who were homozygous for haplotype 1 (8 from the first series and 3 from the second series). Most of these patients had vitiligo and at least one other autoimmune disease. The sequenced regions included a contiguous segment of 69.1 kb that spanned the extended *NALP1* promoter region and exons 1, 2, and 3, as well as 11 individual segments containing exons 4 through 18 with 90 to 500 bp of adjacent intronic sequences. We sequenced several small introns completely. The regions that we sequenced define the five known alternatively spliced isoforms of *NALP1* messenger RNA (mRNA); approximately 77% of the sequence was

determined by analyzing both DNA strands. Nucleotide positions were obtained from the human genome sequence for chromosome 17 from the National Center for Biotechnology Information (NCBI) (Build 36).

The predicted effect of the substitution of histidine for leucine at position 155 (Leu155→His) on the secondary structure of the NALP1 protein was assessed with the use of the protein structure prediction server (PSIPRED).[16] We investigated the potential effects of both alleles of all promoter-region variants on transcription-factor binding motifs predicted with the use of the Transcription Element Search System (TESS)[17] and rVista 2.0 software.[18]

STATISTICAL ANALYSIS

Details on preliminary analyses, genetic-linkage analyses, and conditional logistic-regression analysis are given in the Supplementary Appendix. We tested for Hardy–Weinberg equilibrium in founders (the earliest specified persons in lineages) and in persons not in the lineage, such as spouses, in all 114 families. Calculation of linkage disequilibrium between markers of the *NALP1* region was carried out with Haploview software,[19] version 3.32. We also calculated the association of each marker with vitiligo or with an expanded autoimmune phenotype considering as affected persons with any autoimmune or autoinflammatory disease associated with vitiligo using the family-based association test,[15] version 1.5.5, and the pedigree disequilibrium test,[14] version 5.1, which provides a combined statistic accounting for allele transmission to both affected offspring and unaffected offspring. Haplotype-based transmission-disequilibrium statistics were calculated with the use of the family-based association test, version 1.5.5. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

GENETIC-LINKAGE ANALYSIS

We previously reported[10] genetic linkage between a locus on chromosome 17p13 and multiple autoimmune disease associated with vitiligo, a result obtained by genotyping microsatellite markers across the genome in 51 extended families (the first series). In other families with vitiligo only, there was no linkage to this chromosomal region.[10] With the use of these same data but an improved error-checking algorithm,[20] the maximum multipoint lod score was 4.59 ($P=2.14\times10^{-6}$) — an improvement on the previously obtained lod score of 4.00.[10] The multipoint maximum lod score occurred at approximately 4.3 cM on chromosome 17p; the linkage region encompassing a 1-lod reduction from the maximum (thus, the region that probably contains the causal gene) spanned 11.3 cM (Fig. 1A).

FAMILY-BASED ASSOCIATION STUDIES

There are approximately 80 known or predicted genes within the 11.3-cM region spanning the linkage peak on chromosome 17p. To determine which of these genes is most likely to contribute to multiple autoimmune disease associated with vitiligo, we genotyped the families in the first series for the 177 SNPs spanning the linkage peak. The frequencies of all SNPs in founders and spouses who had married into the family were consistent with Hardy–Weinberg equilibrium. Genetic-linkage analysis incorporating both SNP and microsatellite data did not result in a significant narrowing of the linkage peak (results not shown). We then tested for an allelic association of these SNPs with vitiligo alone and with the entire group of autoimmune and autoinflammatory diseases associated with vitiligo, considering a family member with any of these diseases as "affected." We used both the pedigree disequilibrium test[14] and the family-based association test,[15] which yielded generally similar results (Table 2 in the Supplementary Appendix). Both tests showed that 23 SNPs were associated with the vitiligo phenotype, with the expanded autoimmune and autoinflammatory disease phenotype, or with both, including a cluster of five adjacent SNPs — rs2301582, rs9889625, rs3926687, rs2733359, and rs878329 — spanning a 117-kb region (Fig. 1B).

To assess the reproducibility of these candidate association signals, we carried out an independent analysis in which we genotyped the 23 significant SNPs in a second series of 63 extended families with multiple autoimmune disease associated with vitiligo. The results of this analysis provided support for an association of three of the five adjacent SNPs — rs3926687, rs2733359, and rs878329 — with the vitiligo phenotype and with the expanded autoimmune and autoinflammatory disease phenotype, both in the second series and in all 114 families (Fig. 1B, and Table 2 in the Supplementary Appendix).

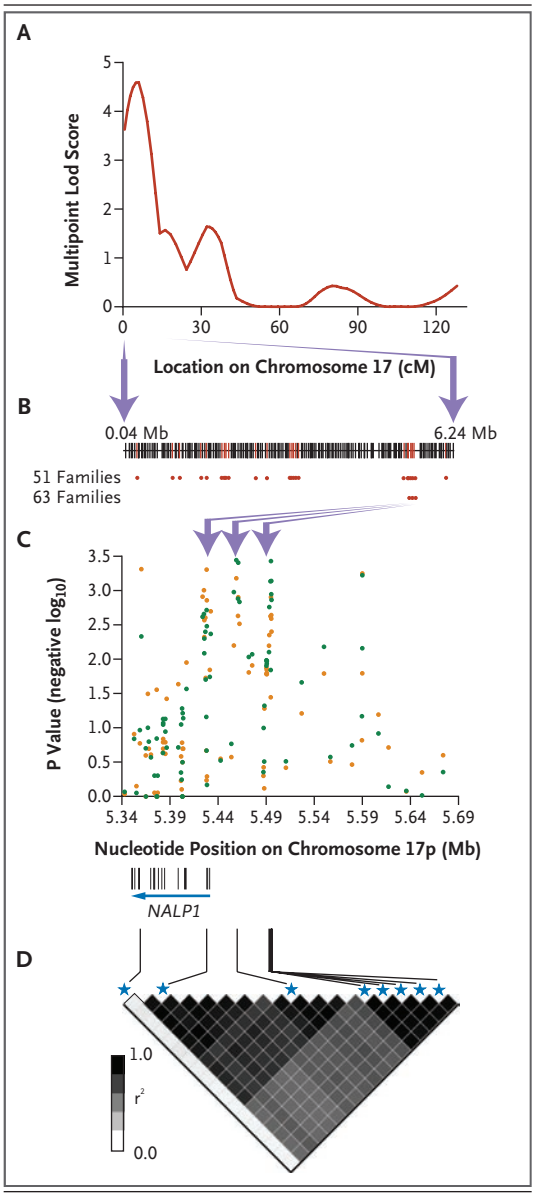

Figure 1. Association of Autoimmune Disease with the Chromosome 17 Linkage Region and *NALP1*.

Panel A shows the nonparametric multipoint lod scores for markers across chromosome 17 for 51 multiplex families with vitiligo-associated multiple autoimmune and autoinflammatory disease. Panel B shows the distribution of 177 single-nucleotide polymorphisms (SNPs) genotyped across the 17p linkage region, from 0.04 to 6.24 Mb, in the same 51 families. The 23 SNPs that were each significantly associated with disease in these 51 families (red bars and red circles) were then genotyped in a replicate series of 63 similar families (red circles indicate SNPs that were significantly associated with disease in these families) (see Table 2 in the Supplementary Appendix). Panel C shows the negative $\log_{10}$ of the P values from the pedigree disequilibrium test for the 85 variants (83 SNPs and 2 insertion–deletion polymorphisms) genotyped across the *NALP1* region of chromosome 17p in all 114 families. Green circles indicate the vitiligo phenotype, and orange circles indicate the extended autoimmune and autoinflammatory disease phenotype. The 18 exons of the *NALP1* structural gene are indicated by the black bars (transcriptional orientation shown from right to left, blue arrow). Panel D shows pairwise r^2 values for linkage disequilibrium (with darker boxes indicating stronger disequilibrium) among the 19 of the 85 *NALP1* region markers shown in Panel C that were most consistently associated with disease and thus were used in conditional logistic-regression analyses, graphed against the physical positions of the markers. Stars indicate markers for which potential independent effects could not be excluded through regression analysis.

These three high-risk SNPs span a 61-kb segment that includes the proximal coding region and the extended promoter region of *NALP1*, which encodes a key regulator of the innate immune system (Fig. 1C). The genomic region around *NALP1* is gene-sparse; SNPs located downstream of *NALP1* were not associated with disease, and the closest upstream gene, *KIAA0523*, is more than 486 kb toward the centromere from *NALP1*. The results of the family-based association test showed that a preliminary haplotype defined by these three SNPs (haplotype 1) had the most significant association with both the vitiligo phenotype and the expanded autoimmune and autoinflammatory disease phenotype in the first series ($P=0.01$ and $P=0.009$, respectively), in the second series ($P=0.03$ and $P=0.02$, respectively), and in both series combined ($P<0.001$ for both comparisons; odds ratio for vitiligo, 1.85; 95% confidence interval [CI], 1.25 to 2.71; odds ratio for autoimmune and autoinflammatory disease, 1.79; 95% CI, 1.25 to 2.56). Of the 177 SNPs initially tested, 98 clustered into 21 blocks of linkage disequilibrium. With regard to correction for multiple testing, the 177 SNPs constituted approximately 100 independent tests (21 blocks of linkage disequilibrium and the 79 remaining SNPs), with a corrected P value of 0.04 for vitiligo-associated autoimmune and autoinflammatory diseases.

SEQUENCE ANALYSIS AND HIGH-DENSITY ASSOCIATION ANALYSIS

Sequence analysis of *NALP1* and its extended promoter region was performed in 11 persons with vitiligo who were homozygous for haplotype 1 and in 4 unaffected heterozygotes who had transmitted haplotype 1 to at least one affected offspring. The analysis yielded a total of 261 sequence vari-

ants (Table 3 in the Supplementary Appendix), 54 of which were newly discovered. We also identified a segment of 524 bp that was missing from the NCBI human chromosome 17 sequence, immediately after nucleotide 5,466,866.

To define more precisely the *NALP1* genomic region that confers susceptibility to autoimmune and autoinflammatory disease, we genotyped all 114 families for 78 additional SNPs (identified by means of sequence analysis) (Fig. 1C) and two small insertion–deletion polymorphisms (Table 4 in the Supplementary Appendix), selected on the basis of their physical positions, HapMap tag-SNP status, minor allele frequencies, and potential functional significance. The genotype frequencies of all variants tested were consistent with Hardy–Weinberg equilibrium and were similar in the two series (data not shown). As shown in Figure 1C, many *NALP1* region variants were associated with vitiligo (with P values ranging from 0.048 to <0.001 and odds ratios ranging from 1.39 to 2.08) or with associated autoimmune and autoinflammatory disease (with P values ranging from 0.04 to <0.001 and odds ratios ranging from 1.25 to 1.93), in a pattern broadly distributed across the proximal portion of the *NALP1* structural gene and its extended promoter region. In general, we observed a stronger association for the expanded autoimmune and autoinflammatory disease phenotype than for the smaller vitiligo subgroup (Table 4 in the Supplementary Appendix).

Apparent associations of disease with multiple markers in the *NALP1* region may reflect multiple independent causal variants or may be a consequence of linkage disequilibrium between multiple markers and one true causal variant. The alignment of the genomic positions (Fig. 1C) and the linkage-disequilibrium pattern (Fig. 1D and Table 2) of the 19 *NALP1* region markers (17 SNPs and 2 insertion–deletion polymorphisms) for which an association with disease was replicated in the two series (by means of the pedigree disequilibrium test and the family-based association test) suggested that at least two markers might be independently associated with disease. To distinguish markers that might reflect independent variants from those that merely reflect linkage disequilibrium, we carried out conditional logistic-regression analyses[21] of these 19 markers (Table 2, and Table 5 in the Supplementary Appendix). On the basis of this analysis, three markers (rs6502867, rs8182352, and rs4790797) had the largest individual effects both for the expanded autoimmune and autoinflammatory disease phenotype (odds ratios, 1.93, 1.81, and 1.82, respectively; P<0.001 for all three markers) and for the vitiligo phenotype (odds ratios, 2.08, 2.01, and 2.01, respectively; P< 0.001 for all three markers). The inclusion of rs6502867 significantly improved the fit of logistic-regression models that included any 1 of the 18 other markers; conversely, the fit of the model including rs6502867 was significantly improved by the inclusion of any 1 of 15 of the 18 other markers (Table 5 in the Supplementary Appendix). These results provide further support for the existence of at least two independent variants in the *NALP1* region associated with the risk of disease: one variant tagged by rs6502867 and the other located further upstream, in the proximal coding region or in the transcriptional promoter.

The markers rs878329, rs7223628, rs8182352, and rs4790796 are in almost perfect linkage disequilibrium with rs4790797 (Table 6 in the Supplementary Appendix), indicating that these five SNPs, which span only 2107 bases, all represent the same signal. The colinearity among the five markers precludes logistic-regression analyses that include any two of them. Inclusion of rs4790797 significantly improved the fit of models that included any 1 of the 14 remaining markers, except for rs12150220 and rs2670660, whereas none of the 14 remaining markers, except for rs6502867, improved the fit of the model that included rs4790797. These results suggest that an association of 11 of the 14 markers (rs961826, rs11078575, rs1877658, rs925597, rs925598, rs3926687, the 12-bp deletion, rs2733359, rs35658367, rs2716914, and rs8182354) with disease may be secondary to linkage disequilibrium with the cluster of 5 SNPs represented by rs4790797. Overall, the marker most significantly associated with disease was rs4790797, but it could not be distinguished from rs12150220 and rs2670660 in the logistic-regression analysis (Table 5 in the Supplementary Appendix). The results for the vitiligo phenotype were similar to those for the expanded autoimmune and autoinflammatory disease phenotype, except that the association of rs12150220 with vitiligo also appeared to be secondary to linkage disequilibrium with rs4790797.

Thus, we detected at least two independent signals associated with autoimmune and autoinflammatory diseases: one located within the *NALP1* structural gene, tagged by SNP rs6502867, and at

Table 2. Association of 19 *NALP1* Variants with Vitiligo and Autoimmune and Autoinflammatory Disease in 114 Multiplex Families.*

Variant/Allele	P Value			Odds Ratio (95% CI)†
	Pedigree Disequilibrium Test	Family-Based Association Test	Conditional Logistic-Regression Analysis	
Autoimmune and autoinflammatory disease				
rs6502867/A	<0.001	0.002	<0.001	1.93 (1.32–2.88)
rs961826/A	0.001	0.001	0.008	1.60 (1.14–2.24)
rs12150220/A	0.001	0.001	0.003	1.66 (1.19–2.31)
rs11078575/C	0.003	0.002	0.02	1.50 (1.08–2.08)
rs1877658/T	0.003	0.005	0.04	1.40 (1.01–1.93)
rs925597/A	<0.001	<0.001	0.005	1.62 (1.16–2.27)
rs925598/A	0.001	0.002	0.004	1.63 (1.17–2.26)
rs3926687/T	0.002	0.003	0.006	1.59 (1.15–2.21)
12-bp deletion‡	0.006	0.01	0.01	1.51 (1.10–2.09)
rs2670660/C	<0.001	<0.001	0.003	1.68 (1.20–2.35)
rs2733359/G	0.002	0.001	0.005	1.64 (1.17–2.30)
rs35658367/ATGA	0.001	0.001	0.004	1.64 (1.17–2.31)
rs2716914/C	0.003	0.004	0.02	1.49 (1.08–2.07)
rs878329/G	0.005	0.001	0.003	1.63 (1.17–2.26)
rs7223628/G	0.002	<0.001	0.002	1.68 (1.21–2.35)
rs8182352/G	0.001	<0.001	<0.001	1.81 (1.28–2.56)
rs4790796/A	0.003	<0.001	0.001	1.73 (1.24–2.42)
rs4790797/T	0.002	<0.001	<0.001	1.82 (1.29–2.56)
rs8182354/A	0.004	0.001	0.002	1.69 (1.22–2.36)
Vitiligo				
rs6502867/A	0.005	0.004	<0.001	2.08 (1.37–3.15)
rs961826/A	0.002	0.003	0.007	1.67 (1.15–2.41)
rs12150220/A	0.002	0.002	0.004	1.69 (1.18–2.41)
rs11078575/C	0.005	0.005	0.02	1.55 (1.09–2.19)
rs1877658/T	0.004	0.007	0.03	1.49 (1.04–2.10)
rs925597/A	0.002	0.003	0.005	1.69 (1.18–2.44)
rs925598/A	0.003	0.003	0.005	1.66 (1.16–2.36)
rs3926687/T	0.004	0.004	0.008	1.61 (1.14–2.29)
12-bp deletion‡	0.001	0.009	0.007	1.66 (1.16–2.36)
rs2670660/C	<0.001	0.001	0.002	1.80 (1.25–2.61)
rs2733359/G	0.001	0.004	0.004	1.75 (1.21–2.53)
rs35658367/ATGA	<0.001	0.001	0.002	1.82 (1.24–2.67)
rs2716914/C	0.002	0.008	0.007	1.64 (1.15–2.35)
rs878329/G	0.002	0.003	0.002	1.75 (1.22–2.51)
rs7223628/G	<0.001	0.002	0.001	1.82 (1.26–2.63)
rs8182352/G	<0.001	<0.001	<0.001	2.01 (1.36–2.95)
rs4790796/A	0.001	0.002	0.001	1.83 (1.26–2.63)
rs4790797/T	<0.001	0.001	<0.001	2.01 (1.39–2.91)
rs8182354/A	0.001	0.002	0.001	1.83 (1.27–2.64)

* The variants listed are those for which the association with disease was replicated by the pedigree disequilibrium test and the family-based association test in both series of families.
† Odds ratios were calculated from the coefficients of the regression equation.
‡ The 12-bp deletion includes nucleotides 5457169 through 5457180 (TATGACTATGTG).

least one other, located within a 64.7-kb linkage disequilibrium block tagged by the nonsynonymous coding SNP rs12150220 (Leu155→His) and six promoter-region SNPs (rs2670660, rs878329, rs7223628, rs8182352, rs4790796, and rs4790797). The significance (P=0.0001) of a model that includes two SNPs, rs6502867 and rs4790797, each of which represents one of the two independent association signals, was greater than that of either individual SNP (P<0.001 for each), and the haplotype carrying both high-risk alleles conferred the highest risk (odds ratio, 2.77; P<0.001) (Table 7 in the Supplementary Appendix).

We assessed the haplotype-specific effects of rs6502867 and rs4790797 (representing the cluster of five SNPs with perfect linkage disequilibrium) by comparing logistic-regression models that included the additive effects of both loci, with and without accounting for linkage phase. We did not detect haplotype-specific effects, which indicates that it makes no difference whether the two variants in the *NALP1* region are cis or trans to one another. We also used logistic-regression models to evaluate the mode of inheritance of risks individually associated with rs6502867 and rs4790797. These analyses favored an additive model with no dominant or recessive effects.

ANALYSIS OF *NALP1* NONSYNONYMOUS CODING-REGION VARIANTS

A total of 15 SNPs in the *NALP1* region predicted nonsynonymous amino acid substitutions in the NALP1 protein (Table 4 in the Supplementary Appendix). These 15 SNPs were included in the second set of 78 SNPs tested for an association with disease in the 114 families (Fig. 1C), and only rs12150220 (Leu155→His) showed evidence of an association, both with vitiligo alone and with autoimmune and autoinflammatory diseases (P=0.002 and P=0.001, respectively, by the pedigree disequilibrium test). Leu155→His is a nonconservative substitution located between the N-terminal pyrin and NACHT domains of the human NALP1 polypeptide. This region contains no known peptide motifs, but its amino acid sequence, including residue Leu155, has been highly conserved through primate evolution, from bush baby to human (Fig. 2) — suggesting that the region is critical to protein function. Indeed, the predicted secondary structure of the region has been even more highly conserved than its sequence.

NALP1 PROMOTER-REGION VARIANTS

We observed a total of 205 variants in the extended promoter region, all of which were assessed for predicted effects on transcription-factor binding motifs. Five of the six tightly linked SNPs in the promoter region that were associated with disease were found to affect high-probability mammalian transcription-factor binding sites (Table 3). Furthermore, rs2670660 occurs within a segment that is remarkably conserved in the human, chimpanzee, macaque, bush baby, cow, mouse, and rat, suggesting that this variant is functionally significant. It alters predicted binding motifs for the transcription factors HMGA1 [HMG-I(Y)] and MYB. MYB regulates transcription during the differentiation, proliferation, and apoptosis of erythroid, myeloid, and lymphoid cell lineages. Whether any of these sequence variants

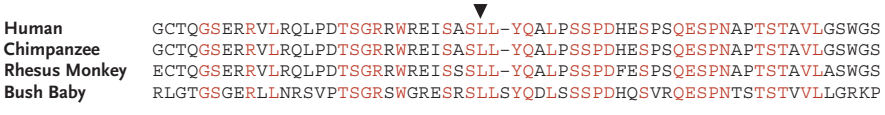

Figure 2. Alignment of Predicted Primate NALP1 Amino Acid Sequences Surrounding the Leu155→His Substitution (Arrowhead).

Residues in red are completely conserved among known primates; those in black are not. Dashes indicate gaps introduced to maximize sequence alignments. The NALP1 sequence of humans (*Homo sapiens*) is from ENSG00000091592 from the National Center for Biotechnology Information genome sequence (Build 36). The sequence of the chimpanzee (*Pan troglodytes*) is from ENSPTRG00000008637 from the PanTro, version 2.1, database. The sequence of the rhesus monkey (*Macaca mulatta*) was obtained through manual annotation of the entry for ENSMMUG00000030453 in the MMUL, version 1.0, database. The sequence of the bush baby (*Otolemur garnettii*) was obtained through manual assembly and annotation of data from scaffolds 14080 and 113389 from the bush baby, version 1.0, prerelease database. NALP1 in the cow, dog, mouse, rat, and ground squirrel lacks the entire NH_4-terminal segment found in the primate protein, and sequences orthologous to NALP1 could not be identified in any other early-release genomic databases.

Table 3. Predicted Transcription-Factor Binding Motifs Affected by *NALP1* Promoter-Region SNPs Associated with Disease.*

SNP	Nucleotide Position†	Transcription Factor	L_a	L_q
rs2670660	5,459,730			
T		HMGA1 [HMG-I(Y)]	22.00	0.917
C (high risk)		MYB	12.00	1.000
rs878329	5,493,974			
C		PEBP2	18.00	0.900
G (high risk)		—		
rs7223628	5,495,192			
A		AP-1	18.00	0.900
		NFAT-1	14.00	1.000
G (high risk)		TCF2 (HNF-1B)	14.00	1.000
		PU.1	14.00	1.000
rs8182352	5,495,711			
A		—		
G (high risk)		PR	12.00	1.000
rs4790797	5,496,043			
C		FOXF1	24.00	0.923
T (high risk)		—		

* The five SNPs are among the six promoter-region SNPs whose potential independent effects could not be ruled out by conditional logistic-regression analyses. L_a denotes the log-likelihood score. L_q, a measure of the goodness-of-fit of the DNA sequence to the consensus binding motif, was calculated by dividing L_a by the maximum L_a possible for the site model; the best possible L_q was 1.000.

† The *NALP1* exon 1 (the site of the start of translation) begins at nucleotide 5,428,550.

affects *NALP1* transcription in humans requires further investigation.

DISCUSSION

Our study shows that variants of *NALP1* confer susceptibility to autoimmune and autoinflammatory diseases that are associated with vitiligo. Confirmation of this finding will require additional studies in other patient groups, including analysis of the extent to which *NALP1* is specifically involved in the component autoimmune disorders. Furthermore, the SNPs that we have implicated may not be the causal variants; identification of such variants will require the demonstration of specific effects on *NALP1* transcription, mRNA, or protein function.

NALP1, also known as *CARD7*, *DEFCAP*, and *NAC*, is thought to mediate activation of the innate immune system in response to so-called pathogen-associated molecular patterns such as bacterial peptides.[22-24] *NALP1* is widely expressed at low levels but is expressed at a high level in immune cells, particularly T cells and Langerhans' cells,[25,26] patterns that are consistent with the particular involvement of NALP1 in skin autoimmunity. NALP1 recruits the adapter protein ASC, caspase 1, and caspase 5 to a complex termed the NALP1 inflammasome,[23,24,26] which activates the proinflammatory cytokine interleukin-1β. Serum interleukin-1β levels are elevated in patients with generalized vitiligo,[27] suggesting the involvement of this pathway in the pathogenesis of disease. NALP1 also appears to play a role in cellular apoptosis, its overexpression stimulating caspase-mediated apoptosis in a variety of cell types.[22,28,29]

Mutations in at least two other *NALP*-related genes involved in the innate immune system are associated with autoinflammatory diseases. Variants in *NOD2/CARD15* are associated with inflammatory bowel disease[30,31] and the Blau syndrome.[32] Mutations in *NALP3/CIAS1*, a homologue of *NALP1* and a component of the NALP3 inflammasome,[24,26] result in several autoinflammatory phenotypes, including the cold autoinflammatory syndrome, the Muckle–Wells syndrome, and neonatal-onset multisystem inflammatory disease.[33-35] Administration of an interleukin-1β inhibitor[36,37] or a caspase-1 inhibitor[38] ameliorates symptoms in patients with these disorders. If the association between *NALP1* and autoimmune and autoinflammatory diseases is confirmed, and if *NALP1* variants are found to result in the activation of interleukin-1β, then inhibitors of interleukin-1β and caspase might be effective in the treatment or prevention of *NALP1*-associated autoimmune and autoinflammatory diseases.

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