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Fine-Mapping of Vitiligo Susceptibility Loci on Chromosomes 7 and 9 and Interactions with *NLRP1* (*NALP1*)

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Generalized vitiligo is the most common pigmentation disorder, the result of autoimmune loss of melanocytes from the skin and hair, with a high frequency of other autoimmune diseases in vitiligo patients and their relatives. We previously reported the linkage signals on chromosomes 1, 7, and 17 in Caucasian families with generalized vitiligo and associated autoimmune diseases and identified the risk loci of chromosomes 17 and 1 as *NLRP1* (*NALP1*) and *FOXD3*, respectively. Here, we describe fine-scale genetic association analyses in two independent series of Caucasian multiplex families, refining localization of the chromosome 7 locus and a locus on chromosome 9. Three susceptibility signals, represented by single-nucleotide polymorphisms (SNPs) rs6960920 in 7p13, rs734930 in 7q11, and rs4744411 in 9q22, were significantly associated with vitiligo and other autoimmune diseases. We also detected significant three-way interaction effects of chromosome 7 SNP rs6960920, chromosome 9 SNP rs4744411, and *NLRP1* SNP rs6502867 on both the vitiligo phenotype and an expanded autoimmune disease phenotype, and significant three-way interaction effects of both chromosome 7 SNPs and *NLRP1* SNP rs6502867 on the vitiligo phenotype. These support the validity of the chromosomes 7 and 9 linkage/association signals and underscore the utility of gene–gene interaction analysis in characterizing the genetic effects of candidate association signals.

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INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder, in which progressive, patchy loss of pigmentation from the skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). The most common pigmentation disorder, vitiligo, occurs in about 0.4% of European Caucasians (Howitz *et al.*, 1977), with similar prevalence in other populations (Spritz, 2007, 2008). First-degree relatives of affected patients have ~6 to 7% risk of vitiligo, and concordance in monozygotic twins is 23% (Alkhateeb *et al.*, 2003). About 25–30% of generalized vitiligo patients manifest at least one other autoimmune disease, particularly autoimmune thyroid disease (Graves' disease and autoimmune hypothyroidism), rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus; these same disorders also occur in increased frequency in patients' first-degree relatives (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). Thus, this complex of associated autoimmune diseases likely results from the interaction of multiple disease susceptibility variants (Majumder *et al.*, 1993; Nath *et al.*, 1994; Sun *et al.*, 2006), some predisposing to a general autoimmune diathesis and others to specific autoimmune diseases.

A number of candidate genes have been reported as associated with generalized vitiligo, including loci in the major histocompatibility complex, *ACE*, *CAT*, *CTLA4*, *COMT*, *ESR*, *GCH1*, *MBL*, *PTPN22*, *VDR*, and others (Spritz, 2007, 2008). Most of these studies reported only marginally significant associations, often with no correction for multiple testing, and several were not replicated by subsequent studies (Spritz, 2007, 2008).

We previously reported significant linkage (logarithm of the odds (LODs) $\geqslant$3.0) of generalized vitiligo to loci on chromosomes 1, 7, 8, and 17 and suggestive linkage (LODs $\geqslant$2.0) to loci on chromosomes 9, 13, 19, and 22 in an analysis of 102 multiplex generalized vitiligo families. The linkage signals on chromosomes 1, 7, and 17 derived principally from the subset of 51 families segregating both vitiligo and additional vitiligo-associated autoimmune diseases, whereas the linkage signal on chromosome 8 was

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Abbreviations: CI, confidence interval; CLRA, conditional logistic regression analysis; FBAT, family-based association test; LD, linkage disequilibrium; NLRP1, *NACHT leucine-rich-repeat protein; OR, odds ratio; PDT, pedigree disequilibrium test; SNP, single-nucleotide polymorphism*

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derived principally from the subset of families segregating only vitiligo (Spritz *et al.*, 2004). Subsequently, we identified the chromosome 17 vitiligo-autoimmunity susceptibility locus as *NLRP1* (NACHT leucine-rich-repeat protein) (Jin *et al.*, 2007), encoding NALP1, a key regulator of the innate immune system, and the linkage signal on chromosome 1 derived primarily from a single large family (Alkhateeb *et al.*, 2002), in which generalized vitiligo co-segregated with a promoter variant in *FOXD3* (Alkhateeb *et al.*, 2005).

The purpose of the current study was to refine localization of these putative vitiligo-autoimmune disease susceptibility loci. We first carried out improved genetic linkage analysis, increasing linkage support for the chromosomes 7 and 9 loci, whereas greatly reducing support for loci on chromosomes 13, 19, and 22. We then carried out fine-scale genetic association analyses through the chromosomes 7 and 9 linkage regions in the aforementioned 51 families that segregated both vitiligo and additional associated autoimmune diseases, detecting apparent association for 17 single-nucleotide polymorphisms (SNPs) from the chromosome 7 linkage region and 8 SNPs from the chromosome 9 linkage region. We then re-tested these SNPs in an independent series of 63 vitiligo-autoimmune disease families, with a joint analysis showing significant associations on both chromosomes after correction for multiple testing. We next investigated potential genetic interactions among these variants and with the known vitiligo susceptibility variants in *NLRP1*, detecting significant interactions that underscore the polygenic, complex nature of vitiligo and associated autoimmune diseases.

RESULTS

Genetic linkage analysis

We first reassessed the evidence for genetic linkage, filtering the previous microsatellite data (Spritz *et al.*, 2004) using an improved error-checking routine implemented in Merlin 1.1.2 (Abecasis *et al.*, 2002), and then re-analyzing linkage in the total 102 multiplex vitiligo families and the subset of 51 families that also segregated other autoimmune diseases using Allegro 1.2c (Gudbjartsson *et al.*, 2000). With these enhanced analyses, the maximum multipoint LOD for chromosome 7 increased to 4.31 ($P = 6.07 \times 10^{-6}$) for the total 102 families and 4.01 ($P = 1.52 \times 10^{-6}$) for the 51 vitiligo-autoimmune disease families; the LOD maximum was located at 89.4 cM and the 1-LOD interval spanned ~33.3 cM in 7q21. Similarly, a locus on chromosome 9, which previously showed only suggestive evidence for linkage in the total 102 vitiligo families (Spritz *et al.*, 2004), now yielded a maximum multipoint LOD score of 2.37 ($P = 2.24 \times 10^{-4}$) for the total 102 families and 3.18 ($P = 6.35 \times 10^{-5}$) for the 51 vitiligo-autoimmune disease families; the LOD maximum was at 88.1 cM and the 1-LOD interval spanned ~17.7 cM in 9q12-q22. In contrast, support for putative vitiligo susceptibility loci on chromosomes 8, 13, 19, and 22 was reduced substantially, below the threshold for suggestive linkage (LOD 1.9); accordingly, these three signals were not pursued further.

Family-based association studies

To refine localization of the chromosome 7 and 9 vitiligo-autoimmunity susceptibility loci, we next carried out family-based association analyses of high-density SNPs genotyped through the 1-LOD linkage intervals. We genotyped 333 members of the aforementioned 51 vitiligo-autoimmune disease families for 867 SNPs spanning the chromosome 7 linkage region and 304 SNPs spanning the chromosome 9 linkage region, respectively, capturing 38.6 and 27.2% of the common variation (minor allele frequency >0.1, $r^2>0.5$) in these regions (NCBI Genome Build 35). Two SNPs from chromosome 7 and four from chromosome 9 deviated from the Hardy–Weinberg equilibrium ($P<0.005$) in unrelated founders and were excluded from further analyses. We used both the pedigree disequilibrium test (PDT) (Martin *et al.*, 2000) and the family-based association test (FBAT) (Horvath *et al.*, 2004) to assess the remaining markers for association with both vitiligo and with an expanded autoimmune disease phenotype that included family members with either vitiligo or any of the principal vitiligo-associated autoimmune diseases (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005; see Materials and Methods) as ''affected.'' SNPs showing nominal evidence of association ($P<0.05$) with vitiligo and/or the expanded autoimmunity phenotype, of which vitiligo is a subset, in both PDT and FBAT analyses (Figure 1, Tables S1, S2) were then genotyped in 323 family members from an independent series of 63 vitiligo-autoimmune disease families. Altogether, we tested 17 SNPs from the chromosome 7 linkage region and 8 SNPs from the chromosome 9 linkage region for association in the second series of 63 families, corresponding to 14 independent tests after allowing for marker–marker linkage disequilibrium (LD, Figure 2).

Of the 17 SNPs from the chromosome 7 linkage region showing both PDT and FBAT association with the vitiligo phenotype and/or with the expanded autoimmune disease phenotype in the first family series, four (rs6960920, rs2331071, rs2240100, and rs734930) SNPs also showed nominally significant association ($P<0.05$) in PDT analyses of both phenotypes and nominally significant or marginal association in FBAT analyses of the vitiligo phenotype in the second family series (Table 1, Table S1). The PDT *P*-values of these four SNPs in the combined sample of 114 families (series 1 and series 2 together) were significant for the vitiligo phenotype after the Bonferroni correction for the 14 independent tests of chromosome 7 and 9 SNPs (nominal *P*-value <0.00357). SNP rs734930 showed the strongest evidence for association, with significant corrected *P*-values in PDT, FBAT, and conditional logistic regression analysis (CLRA) of the vitiligo phenotype (odds ratio (OR) = 1.85), and PDT analysis of the expanded autoimmune disease phenotype (OR = 1.55). The other three chromosome 7 SNPs (rs6960920, rs2331071, and rs2240100) cluster in a block of strong LD (Figure 2).

To evaluate the relative effects of these four SNPs, we carried out stepwise CLRA of the case–pseudocontrol data set (Cordell and Clayton, 2002) derived from the combined 114 families. The effects of individual SNPs in the three-SNP

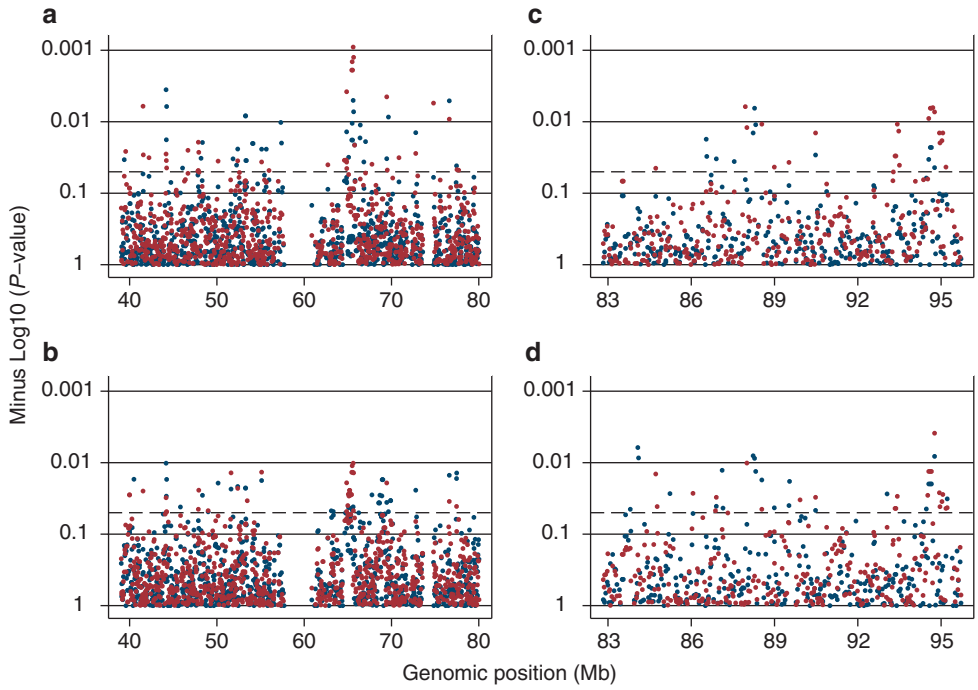

Figure 1. Association of generalized vitiligo and associated autoimmune diseases with chromosome 7 and 9 linkage region SNPs. (**a**) Minus log10 (*P*-values) from the pedigree disequilibrium test (PDT) (Martin *et al.*, 2000) and the family-based association test (FBAT) (Horvath *et al.*, 2004) for the vitiligo phenotype for 867 SNPs genotyped across the chromosome 7 linkage region in the first 51 multiplex families with vitiligo and other autoimmune diseases (series 1). (**b**) Minus log10 (*P*-values) from PDT and FBAT for the expanded autoimmune disease phenotype for 867 SNPs genotyped across the chromosome 7 linkage region in these 51 families. (**c**) Minus log10 (*P*-values) from PDT and FBAT for the vitiligo phenotype for the 304 SNPs genotyped across the chromosome 9 linkage region in these same 51 families. (**d**) Minus log10 (*P*-values) from PDT and FBAT for the expanded autoimmune disease phenotype for the 304 SNPs genotyped across the chromosome 9 linkage region in these 51 families. In each panel, blue circles indicate PDT *P*-values and red circles indicate FBAT *P*-values. The dashed line represents minus log10 (0.05).

cluster were indistinguishable by these analyses, and no haplotype explained the disease associations better than any individual SNPs (data not shown). However, including rs734930 significantly improved the fit of models that included any one of the three other SNPs; conversely, including any one of rs6960920, rs2331071, and rs2240100 significantly improved the fit of models that included rs734930 (Table 2). Thus, at least two independent chromosome 7 variants appear to contribute to genetic association with the vitiligo phenotype (Table 2), one tagged by rs734930 and the other tagged by the three-SNP cluster rs6960920–rs2331071–rs2240100.

Of the eight chromosome 9 SNPs showing both PDT and FBAT association with the vitiligo phenotype and/or the expanded autoimmune disease phenotype in the first family series, four (rs1350564, rs4744369, rs391784, and rs4744411) showed nominally significant or marginal association in FBAT analyses of both phenotypes and nominally significant association in PDT analyses of the expanded autoimmune disease phenotype in the second family series (Table 3, Table S2). The FBAT *P*-values of these four SNPs in the combined sample of 114 families were significant for both phenotypes after the Bonferroni correction for the 14 independent tests. SNP rs4744411 showed the strongest evidence for association with both phenotypes (OR = 2.03 for the vitiligo phenotype and OR = 2.05 for the expanded autoimmune disease phenotype).

As all four of these chromosome 9 SNPs are clustered in a block of strong LD (Figure 2), we again used CLRA to evaluate the relative effects of individual SNPs. Including rs2584806 (vitiligo phenotype) or rs391784 (vitiligo phenotype and expanded autoimmune disease phenotype) significantly improved the fit of models that included rs4744369, but including rs4744369 did not significantly improve the fit of models that included either rs2584806 or rs391784 (Table 4). The individual effects of rs2584806, rs391784, and rs4744411 were indistinguishable by these analyses, and no SNP haplotype explained the disease associations better than did any individual SNPs (data not shown). Thus, the association of rs4744369 with disease is secondary to LD with the other SNPs.

Genetic interaction analyses

We next explored potential genetic interactions among the two independent vitiligo susceptibility signals we detected on chromosome 7p and 7q (tagged by rs6960920 and rs734930, respectively), the signal on 9q (tagged by rs4744411), and the susceptibility variants of *NLRP1* on chromosome 17p that we identified previously (tagged by rs6502867 and rs4790797) (Jin *et al.*, 2007). We carried out CLRA in the combined

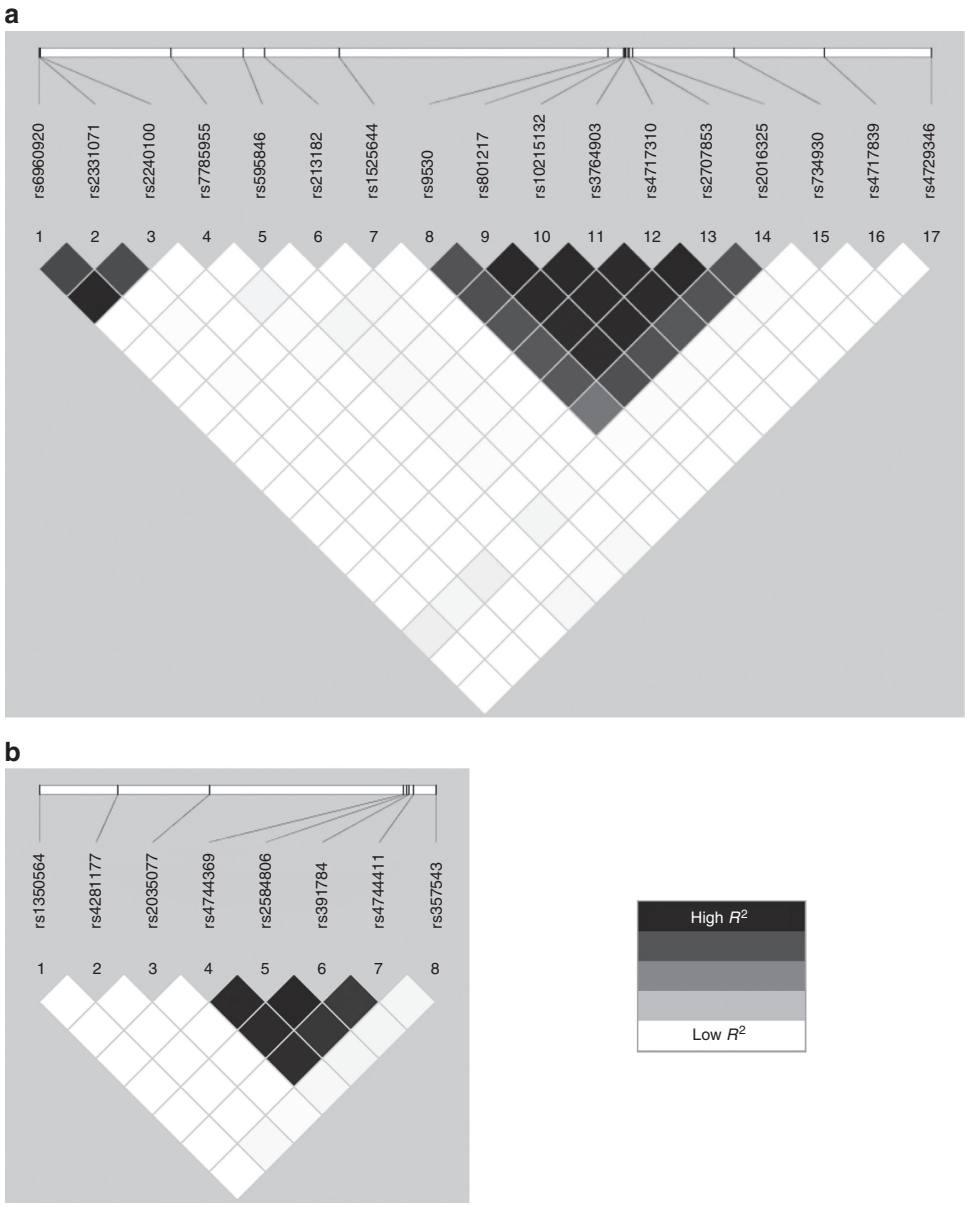

Figure 2. LD patterns among the chromosome 7 and 9 linkage region SNPs. Pairwise r^2 values for LD (22) (darker boxes indicate stronger LD) are shown for the 17 SNPs from the chromosome 7 linkage region (**a**) and the 8 SNPs from the chromosome 9 linkage region (**b**) selected for replication analysis for the vitiligo phenotype and the expanded autoimmune disease phenotype in 63 multiplex families with generalized vitiligo and other autoimmune diseases (series 2).

case–pseudocontrol data set (Cordell and Clayton, 2002; Cordell *et al.*, 2004a) of the five SNPs derived from the combined 114 families, using a three-stage interaction testing framework (Millstein *et al.*, 2006). Analyses were restricted to two- and three-way interactions to avoid over-fitting the data, and tests at higher stage levels were conditional on effects that were already declared significant at lower stage levels to avoid multiple tests of the same effect. The significance threshold for an overall type 1 error rate of 0.05 was determined by the Bonferroni correction based on the number of stages and the number of tests within each stage. As in our previous report of disease associations of *NLRP1* variants in these families (Jin *et al.*, 2007), *P*-values for rs6502867 and rs4790797 met the significance threshold ($P<0.0033$) in stage 1 interaction testing framework analyses of both the vitiligo phenotype (Table 5, $P=0.0022$ and $P=0.0003$, respectively) and the expanded autoimmune disease phenotype (Table 6, $P=0.0005$ and $P=0.0001$, respectively). Consistent with the results of univariate CLRA (Tables 1 and 3), *P*-values for chromosome 7q SNP rs734930 and chromosome 9 SNP rs4744411 met the significance threshold in stage 1 interaction testing framework analyses ($P<0.0033$) of the vitiligo phenotype (Table 5, $P=0.0015$ and $P=0.0011$, respectively), and the *P*-value for chromosome 9 SNP rs4744411 on the expanded autoimmune disease phenotype was very close to significant (Table 6, $P=0.0036$). Although *P*-values for the independent effect of chromosome 7p SNP rs6960920 were not significant for

Table 1. *P*-values for allelic association of chromosome 7 variants with generalized vitiligo and an expanded autoimmune disease phenotype

		51 Family *P*-value		63 Family *P*-value[2]		Combined 114 family *P*-value				
Variant/allele	Position (bp)[1]	PDT[3]	FBAT[4]	PDT[3]	FBAT[4]	PDT[3]	FBAT[4]	CLRA[5]	HRAF[6]	OR (95% CI)[7]
Vitiligo										
rs6960920/G	44,382,597	0.0036	0.0283	0.0117	0.0378	0.0003[8]	0.0066	0.0150	0.558	1.71 (1.11–2.63)
rs2331071/G	44,396,776	0.0179	0.0349	0.0112	0.0539	0.0010[9]	0.0159	0.0124	0.592	1.71 (1.12–2.62)
rs2240100/A	44,433,254	0.0061	0.0444	0.0222	0.0731	0.0012[9]	0.0198	0.0150	0.555	1.71 (1.11–2.63)
rs734930/G	69,658,096	0.0329	0.0045	0.0205	0.0481	0.0032[9]	0.0016[9]	0.0034[9]	0.503	1.85 (1.22–3.76)
Expanded autoimmune disease phenotype[10]										
rs6960920/G	44,382,597	0.0102	0.0310	0.0261	0.1550	0.0017[9]	0.0337	0.0704	0.558	1.41 (0.97–2.05)
rs2331071/G	44,396,776	0.0294	0.0493	0.0121	0.1388	0.0041	0.0519	0.0826	0.592	1.39 (0.96–2.01)
rs2240100/A	44,433,254	0.0171	0.0485	0.0497	0.2572	0.0057	0.0910	0.1147	0.555	1.35 (0.93–1.95)
rs734930/G	69,658,096	0.0360	0.0192	0.0195	0.1416	0.0033[9]	0.0132	0.0129	0.503	1.55 (1.10–2.20)

CI, confidence interval; CLRA, conditional logistic regression analysis; FBAT, family-based association test; OR, odds ratio; PDT, pedigree disequilibrium test.
[1]Nucleotide positions are from National Center for Biotechnology Information (NCBI) (Build 36.3).
[2]One-tailed *P*-value if the family series 2 showed association with the same high-risk allele as in the family series 1.
[3]PDT (Martin *et al.*, 2000).
[4]FBAT (Horvath *et al.*, 2004).
[5]CLRA of the case–pseudocontrol data set (Cordell and Clayton, 2002) derived from the combined 114 families, in which an additive genotypic effect for the high-risk allele of each marker was assumed. We also used logistic-regression models to evaluate the mode of inheritance of risk individually associated with rs6960920 and rs734930, which favored an additive model with no dominant or recessive effects of these markers.
[6]The high-risk allele frequency from the total 114 families.
[7]ORs were calculated from the coefficients of the regression equation.
[8]*P*-value <0.01 after the Bonferroni correction for 14 independent tests.
[9]*P*-value <0.05 after the Bonferroni correction for 14 independent tests.
[10]Expanded autoimmune disease phenotype, in which individuals with any of generalized vitiligo, autoimmune thyroid disease, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, systemic lupus erythematosus, or Addison's disease were considered as affected.[5,6]

either phenotype ($P=0.0141$ for vitiligo, $P=0.1012$ for the expanded autoimmune disease phenotype), there was suggestive evidence of a two-way interaction between rs6960920 and *NLRP1* SNP rs6502867 (Table 5, $P=0.0019$; nominal significance threshold $P=0.0017$), as well as a significant three-way interaction between chromosome 7 SNPs rs6960920 and rs734930 and *NLRP1* SNP rs6502867 (Table 5, $P=0.0009$) for the vitiligo phenotype, and a significant three-way interaction between chromosome 7p SNP rs6960920, chromosome 9 SNP rs4744411, and *NLRP1* SNP rs6502867 for both vitiligo (Table 5, $P=0.0012$) and the expanded autoimmune disease phenotype (Table 6, $P=0.0006$).

DISCUSSION

We have carried out a high-density SNP association study across regions of genetic linkage we previously detected on chromosome 7 and 9 for generalized vitiligo, an autoimmune disease of skin depigmentation. The family-based nature of the study makes the results robust against false-positive associations from cryptic population stratification. The linkage results suggested that loci in these regions might contribute to both generalized vitiligo and to a broader autoimmunity phenotype.

Three signals, tagged by SNPs rs6960920 in 7p13, rs734930 in 7q11, and rs4744411 in 9q22, were significantly associated with generalized vitiligo as well as with a broader autoimmunity phenotype that included vitiligo and other autoimmune diseases commonly associated with vitiligo. These three SNPs also showed significant interactions with *NLRP1* SNP rs6502867, which we previously showed to be associated with vitiligo and other autoimmune diseases in these families (Jin *et al.*, 2007). Both chromosome 7 signals yielded better *P*-values for the vitiligo phenotype than for the expanded autoimmunity phenotype, whereas the chromosome 9 signal yielded generally better *P*-values for the expanded autoimmunity phenotype. Nevertheless, a three-way interaction model that included SNP rs6960920 (7p13), rs4744411 (9q22), and rs6502867 (*NLRP1*) yielded a better *P*-value for the autoimmunity phenotype, suggesting that the 7p13 signal may contribute to broader autoimmune susceptibility.

The rs6960920–rs2331071–rs2240100 SNP cluster is located in a region of chromosome 7p13 that contains two genes, *CAMK2B* and *NUDCD3*. This SNP cluster is located 5′ to *CAMK2B* and 5 kb 3′ to *NUDCD3*, and rs2331071 and rs2240100 are located within *NUDCD3*. *CAMK2B* encodes an apparent member of the serine/threonine protein kinase and Ca(2+)/calmodulin-dependent protein kinase subfamilies that has an essential role in PAF-induced macrophage priming; *CAMK2B* is thus a tenable biological candidate gene

Table 2. Second locus effect tests of the chromosome 7 SNPs in a forward stepwise-regression procedure for the generalized vitiligo and expanded autoimmune disease phenotypes

Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]
Vitiligo											
A	A+B	0.4607	B	B+A	0.8095	—	—	—	—	—	—
A	A+C	Not concave	C	C+A	Not concave	D	D+B	0.0139	B	B+D	0.0030
A	A+D	0.0028	D	D+A	0.0152	D	D+C	0.0152	C	C+D	0.0028
Expanded autoimmune disease phenotype[2]											
A	A+B	0.6435	B	B+A	0.9287	—	—	—	—	—	—
A	A+C	Not concave	C	C+A	Not concave	D	D+B	0.0755	B	B+D	0.0098
A	A+D	0.0109	D	D+A	0.0887	D	D+C	0.1148	C	C+D	0.0107

A, rs66960920; B, rs2331071; C, rs2240100; D, rs734930. ''Not concave'' means it is impossible to obtain a unique estimate of regression coefficients with both variables in the model, which happens when one independent variable is a perfect linear combination of the other, as in the case of perfect LD.
[1]*P*-values from conditional logistic regression analyses.
[2]Expanded autoimmune disease phenotype (see Table 1).

Table 3. *P*-values for allelic association of the chromosome 9 variants with generalized vitiligo and an expanded autoimmune disease phenotype

		51 Family *P*-value		63 Family *P*-value[2]		Combined 114 family *P*-value				
Variant/allele	Position (bp)[1]	PDT[3]	FBAT[4]	PDT[3]	FBAT[4]	PDT[3]	FBAT[4]	CLRA[5]	HRAF[6]	OR (95% CI)[7]
Vitiligo										
rs4744369/T	96,515,217	0.0335	0.0090	0.1559	0.0543	0.0236	0.0023[8]	0.0045	0.411	1.84 (1.21–2.81)
rs2584806/G	96,569,099	0.0229	0.0065	0.1407	0.0484	0.0158	0.0015[8]	0.0028[8]	0.400	1.94 (1.26–3.01)
rs391784/A	96,615,448	0.0229	0.0065	0.0941	0.0314	0.0104	0.0010[8]	0.0017[8]	0.394	2.03 (1.31–3.16)
rs4744411/A	96,728,866	0.0439	0.0072	0.1462	0.0541	0.0307	0.0018[8]	0.0010[8]	0.417	2.03 (1.33–3.10)
Expanded autoimmune disease phenotype[9]										
rs4744369/T	96,515,217	0.0198	0.0133	0.0165	0.0350	0.0177	0.0022[8]	0.0038	0.411	1.89 (1.23–2.91)
rs2584806/G	96,569,099	0.0198	0.0133	0.0279	0.0578	0.0258	0.0033[8]	0.0035[8]	0.400	1.96 (1.25–3.07)
rs391784/A	96,615,448	0.0198	0.0133	0.0185	0.0364	0.0171	0.0022[8]	0.0020[8]	0.394	2.04 (1.30–3.20)
rs4744411/A	96,728,866	0.0082	0.0039	0.0185	0.0278	0.0053	0.0005[10]	0.0012[8]	0.417	2.05 (1.33–3.16)

CI, confidence interval; CLRA, conditional logistic regression analysis; FBAT, family-based association test; OR, odds ratio; PDT, pedigree disequilibrium test.
[1]Nucleotide positions are from the National NCBI Build 36.3.
[2]One-tailed *P*-value if family series 2 showed association with the same high-risk allele as in the family series 1.
[3]PDT (Martin *et al.*, 2000).
[4]FBAT (Horvath *et al.*, 2004).
[5]CLRA of the case–pseudocontrol data set (Cordell and Clayton, 2002) derived from the combined 114 families, in which an additive genotypic effect for the high-risk allele of each marker was assumed. We also used logistic-regression models to evaluate the mode of inheritance of risk associated with rs4744411, which favored an additive model with no dominant or recessive effect.
[6]The high-risk allele frequency from the total 114 families.
[7]ORs were calculated from the coefficients of the regression equation.
[8]*P*-value <0.05 after the Bonferroni correction for 14 independent tests.
[9]Expanded autoimmune disease phenotype (see Table 1).
[10]*P*-value <0.01 the after Bonferroni correction for 14 independent tests.

for generalized vitiligo. *NUDCD3* encodes a protein that functions to maintain the stability of dynein intermediate chain, and appears as an unlikely candidate gene for susceptibility to vitiligo and other autoimmune diseases. SNP rs734930 is located in 7q11.2, within an intron of *AUTS2*, a putative autism candidate gene of unknown

Table 4. Second locus effect tests of chromosome 9 SNPs in a forward stepwise-regression procedure for the generalized vitiligo and expanded autoimmune disease phenotypes

Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]	Null model	Alternative model	*P*-value[1]
Vitiligo											
A	A+B	0.0160	B	B+A	0.1170	B	B+C	Not concave	C	C+B	Not concave
A	A+C	0.0160	C	C+A	0.1186	B	B+D	0.3171	D	D+B	0.9128
A	A+D	0.1976	D	D+A	0.7689	C	C+D	0.5678	D	D+C	0.5195
Expanded autoimmune disease phenotype[2]											
A	A+B	0.0638	B	B+A	0.7056	B	B+C	Not concave	C	C+B	Not concave
A	A+C	0.0359	C	C+A	0.2305	B	B+D	0.2976	D	D+B	0.9558
A	A+D	0.2784	D	D+A	0.9019	C	C+D	0.4441	D	D+C	0.6070

A, rs4744369; B, rs2584806; C, rs391784; D, rs4744411. ''Not concave'' means it is impossible to obtain an unique estimate of regression coefficients with both variables in the model, which happens when one independent variable is a perfect linear combination of the other, as in the case of perfect LD.
[1]*P*-values from conditional logistic regression analyses.
[2]Expanded autoimmune disease phenotype (see Table 1).

Table 5. Wald tests of interactions for the generalized vitiligo phenotype using an interaction testing framework

Stage	Null model	Saturated model	χ^2	d.f.	Significance threshold[1]	*P*-value
1	—	β_1S1	6.03	1	0.0033	0.0141
1	—	β_2S2	10.10	1	0.0033	0.0015
1	—	β_3S3	10.73	1	0.0033	0.0011
1	—	β_4S4	9.37	1	0.0033	0.0022
1	—	β_5S5	13.33	1	0.0033	0.0003
2	β_2S2	β_1S1+β_2S2+β_{12}S1S2	7.75	2	0.0017	0.0227
2	β_3S3	β_1S1+β3S3+β_{13}S1S3	7.67	2	0.0017	0.0216
2	β_4S4	β_1S1+β_4S4+β_{14}S1S4	12.53	2	0.0017	0.0019
2	β_5S5	β_1S1+β_5S5+β_{15}S1S5	8.52	2	0.0017	0.0141
2	β_2S2+β_3S3	β_2S2+β_3S3+β_{23}S2S3	0.15	1	0.0017	0.7032
2	β_2S2+β_4S4	β_2S2+β_4S4+β_{24}S2S4	2.10	1	0.0017	0.1474
2	β_2S2+β_5S5	β_2S2+β_5S5+β_{25}S2S5	0.59	1	0.0017	0.4428
2	β_3S3+β_4S4	β_3S3+β_4S4+β_{34}S3S4	6.09	1	0.0017	0.0136
2	β_3S3+β_5S5	β_3S3+β_5S5+β_{35}S3S5	0.01	1	0.0017	0.9128
2	β_4S4+β_5S5	β_4S4+β_5S5+β_{45}S4S5	0.01	1	0.0017	0.9269
3	β_2S2+β_3S3	β_1S1+β_2S2+β_3S3+β_{12}S1S2+β_{13}S1S3+β_{23}S2S3+β_{123}S1S2S3	16.32	5	0.0017	0.0060
3	β_2S2+β_4S4	β_1S1+β_2S2+β_4S4+β_{12}S1S2+β_{14}S1S4+β_{24}S2S4+β_{124}S1S2S4	20.64	5	0.0017	0.0009
3	β_2S2+β_5S5	β_1S1+β_2S2+β_5S5+β_{12}S1S2+β_{15}S1S5+β_{25}S2S5+β_{125}S1S2S5	10.93	5	0.0017	0.0528
3	β_3S3+β_4S4	β_1S1+β_3S3+β_4S4+β_{13}S1S3+β_{14}S1S4+β_{34}S3S4+β_{134}S1S3S4	20.16	5	0.0017	0.0012
3	β_3S3+β_5S5	β_1S1+β_3S3+β_5S5+β_{13}S1S3+β_{15}S1S5+β_{35}S3S5+β_{135}S1S3S5	10.79	5	0.0017	0.0558
3	β_4S4+β_5S5	β_1S1+β_4S4+β_5S5+β_{14}S1S4+β_{15}S1S5+β_{45}S4S5+β_{145}S1S4S5	16.44	5	0.0017	0.0057
3	β_2S2+β_3S3+β_4S4	β_2S2+β_3S3+β_4S4+β_{23}S2S3+β_{24}S2S4+β_{34}S3S4+β_{234}S2S3S4	7.94	4	0.0017	0.00938
3	β_2S2+β_3S3+β_5S5	β_2S2+β_3S3+β_5S5+β_{23}S2S3+β_{25}S2S5+β_{35}S3S5+β_{235}S2S3S5	6.53	4	0.0017	0.1630
3	β_2S2+β_4S4+β_5S5	β_2S2+β_4S4+β_5S5+β_{24}S2S4+β_{25}S2S5+β_{45}S4S5+β_{245}S2S4S5	5.12	4	0.0017	0.2757
3	β_3S3+β_4S4+β_5S5	β_3S3+β_4S4+β_5S5+β_{34}S3S4+β_{35}S3S5+β_{45}S4S5+β_{345}S3S4S5	6.61	4	0.0017	0.1577

[1]Significance threshold after the Bonferroni correction. SNPs S1, S2, S3, S4, and S5 correspond to SNPs rs6960920, rs734930, rs4744411, rs6502867, and rs4790797, respectively, and the effects of which were assumed to be log-additive. The d.f. value for a test is the difference in the number of parameters between the null and saturated models.

Table 6. Wald tests of interactions for the expanded autoimmune disease phenotype using an interaction testing framework

Stage	Null model	Saturated model	χ^2	d.f.	Significance threshold[1]	*P*-value
1	—	β_1S1	2.69	1	0.0033	0.1012
1	—	β_2S2	5.87	1	0.0033	0.0154
1	—	β_3S3	8.49	1	0.0033	0.0036
1	—	β_4S4	12.11	1	0.0033	0.0005
1	—	β_5S5	14.95	1	0.0033	0.0001
2	—	β_1S1+β_2S2+β_{12}S1S2	6.50	3	0.0017	0.0896
2	—	β_1S1+β3S3+β_{13}S1S3	13.28	3	0.0017	0.0041
2	β_4S4	β_1S1+β_4S4+β_{14}S1S4	7.00	2	0.0017	0.032
2	β_5S5	β_1S1+β_5S5+β_{15}S1S5	3.00	2	0.0017	0.2231
2	—	β_2S2+β_3S3+β_{23}S2S3	12.66	3	0.0017	0.0054
2	β_4S4	β_2S2+β_4S4+β_{24}S2S4	6.54	2	0.0017	0.0380
2	β_5S5	β_2S2+β_5S5+β_{25}S2S5	6.10	2	0.0017	0.0474
2	β_4S4	β_3S3+β_4S4+β_{34}S3S4	12.01	2	0.0017	0.0025
2	β_5S5	β_3S3+β_5S5+β_{35}S3S5	8.86	2	0.0017	0.0119
2	β_4S4+β_5S5	β_4S4+β_5S5+β_{45}S4S5	0.11	1	0.0017	0.7449
3	—	β_1S1+β_2S2+β_3S3+β_{12}S1S2+β_{13}S1S3+β_{23}S2S3+β_{123}S1S2S3	22.16	7	0.0017	0.0024
3	β_4S4	β_1S1+β_2S2+β_4S4+β_{12}S1S2+β_{14}S1S4+β_{24}S2S4+β_{124}S1S2S4	12.98	6	0.0017	0.0433
3	β_5S5	β_1S1+β_2S2+β_5S5+β_{12}S1S2+β_{15}S1S5+β_{25}S2S5+β_{125}S1S2S5	7.89	6	0.0017	0.2463
3	β_4S4	β_1S1+β_3S3+β_4S4+β_{13}S1S3+β_{14}S1S4+β_{34}S3S4+β_{134}S1S3S4	23.68	6	0.0017	0.0006
3	β_5S5	β_1S1+β_3S3+β_5S5+β_{13}S1S3+β_{15}S1S5+β_{35}S3S5+β_{135}S1S3S5	20.38	6	0.0017	0.0024
3	β_4S4+β_5S5	β_1S1+β_4S4+β_5S5+β_{14}S1S4+β_{15}S1S5+β_{45}S4S5+β_{145}S1S4S5	8.37	5	0.0017	0.1370
3	β_4S4	β_2S2+β_3S3+β_4S4+β_{23}S2S3+β_{24}S2S4+β_{34}S3S4+β_{234}S2S3S4	20.49	6	0.0017	0.0023
3	β_5S5	β_2S2+β_3S3+β_5S5+β_{23}S2S3+β_{25}S2S5+β_{35}S3S5+β_{235}S2S3S5	17.56	6	0.0017	0.0074
3	β_4S4+β_5S5	β_2S2+β_4S4+β_5S5+β_{24}S2S4+β_{25}S2S5+β_{45}S4S5+β_{245}S2S4S5	7.75	5	0.0017	0.1706
3	β_4S4+β_5S5	β_3S3+β_4S4+β_5S5+β_{34}S3S4+β_{35}S3S5+β_{45}S4S5+β_{345}S3S4S5	13.50	5	0.0017	0.0191

[1]Significance threshold after Bonferroni correction. SNPs S1, S2, S3, S4, and S5 correspond to SNPs rs6960920, rs734930, rs4744411, rs6502867, and rs4790797, respectively, and the effects of which were assumed to be log-additive. The d.f. value for a test is the difference in the number of parameters between the null and saturated models.

function; and SNP rs4744411 is located in chromosome 9q22, within an intron of *C9orf3*, a predicted gene of unknown function. Further studies of these to identify the specific causal genes and causal disease susceptibility variants will elucidate new biological pathways that mediate susceptibility to generalized vitiligo and associated autoimmune diseases, facilitating the development of new approaches to the treatment or prevention of these disorders.

MATERIALS AND METHODS

Subjects

Study subjects included 656 individuals from 114 Caucasian extended families with generalized vitiligo and additional autoimmune diseases from the United States and the United Kingdom. Families were selected based on the joint criteria of multiple family members affected with generalized vitiligo, and at least one family member affected with at least one of the other autoimmune diseases that are epidemiologically associated with generalized vitiligo (autoimmune thyroid disease, rheumatoid arthritis, adult-onset autoimmune diabetes mellitus, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease) (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). We studied two family series. Series 1 included 51 extended families (333 individuals) that we had previously used to map a vitiligo-autoimmune disease locus to chromosome 1, 7, 8 and 17 (Spritz *et al.*, 2004), and series 2 included 63 similar independent families (323 individuals). All families from series 1, and about half from series 2, are presented in Laberge *et al.*, 2005.

All available relevant affected and unaffected family members completed a detailed questionnaire providing clinical history of about 50 autoimmune, autoinflammatory, and immune-related diseases. All data were reviewed by the study investigators, and most family members (affected and unaffected) were personally examined by one of the study staff. Clinical details of the 114 study families, including co-morbid autoimmune diseases, have been described previously (Jin *et al.*, 2007). This study was approved by

the Colorado Multiple Institutional Review Board (COMIRB) and the South Thames Regional Multicentre Research Ethics Committee (MREC), and the Declaration of Helsinki protocols were followed. Written informed consent was obtained from all study participants.

Genotyping

DNA was prepared from peripheral blood or saliva using the Puregene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN) or Oragene Collection Kit (Oragene; DNA Genotek, Ottawa, Ontario, Canada) respectively. We used the Illumina genotyping service (Illumina, San Diego, CA) to genotype the same 51 families (series 1) we previously used for genetic linkage analysis (Spritz *et al.*, 2004), assaying 867 SNPs spanning the chromosome 7 linkage region (47 kb average spacing) and 304 SNPs spanning the chromosome 9 linkage region (42 kb average spacing). We next carried out a replication study in an independent series of 63 families (series 2) collected using the same criteria as series 1, genotyping the 17 SNPs from the chromosome 7 linkage region and the 8 SNPs from the chromosome 9 linkage region that yielded nominally significant association ($P<0.05$) by both PDT (Martin *et al.*, 2000) and FBAT (Horvath *et al.*, 2004) in series 1. Genotyping of the total 25 SNPs in series 2 was performed using the ABI Prism SNaPshot Multiplex kit on an ABI 3130XL DNA analyzer (Applied Biosystems, Foster City, CA) with HPLC-purified oligonucleotides (Operon, Huntsville, AL). To compare genotyping results obtained using the two platforms, we used the ABI system to re-genotype nine SNPs in the 333 individuals of series 1 and obtained a concordance rate of 99.8%.

Statistical analyses

Preliminary analyses. We assessed the inheritance of each marker in all families using PedCheck (O'Connell and Weeks, 1998) to test for inconsistencies owing to non-paternity, new mutations, or other errors.

Genetic linkage analysis. Analyses of our previous microsatellite data (Spritz *et al.*, 2004) were carried out by applying the improved error-checking routine implemented in Merlin 1.0.0 (Abecasis *et al.*, 2002) and then using Allegro 1.2c software (Gudbjartsson *et al.*, 2000) to calculate LOD scores using an exponential model under the S_{pairs} allele-sharing statistic.

Hardy–Weinberg equilibrium, LD, and disease association analyses. Testing for the Hardy–Weinberg equilibrium in founders and persons not in the line of descent, such as spouses, in all 114 families, and calculation of LD between *NLRP1* region markers, was carried out using Haploview (Barrett *et al.*, 2005) version 4.1. Calculation of the association of each marker with generalized vitiligo or with an expanded autoimmune phenotype that included any of generalized vitiligo, autoimmune thyroid disease, rheumatoid arthritis, adult-onset autoimmune diabetes mellitus, psoriasis, pernicious anemia, systemic lupus erythematosus, or Addison's disease was carried out using PDT (Martin *et al.*, 2000) and FBAT (Horvath *et al.*, 2004). Haplotype-based transmission-disequilibrium statistics were calculated using FBAT.

To distinguish independent associations from secondary associations owing to LD, we applied CLRA (Cordell and Clayton, 2002) to the case–pseudocontrol data set derived from the combined 114 families. To allow for correlation between multiple affected individuals within a single pedigree, we clustered cases and matched pseudocontrols by pedigree and used robust the Huber–White variance estimators to assess the significance of each association (Cordell, 2004b). We assumed an additive genotypic effect for the high-risk allele of each locus. ORs were estimated using the coefficients from the regression equations for individual markers. As a test of the independent effect of a given locus conditioned on the effect of another locus, we compared the fit of a model containing both loci to a model containing only the conditioning locus (Cordell and Clayton, 2002). To test interactions among the chromosome 7 SNPs rs6960920 and rs734930, the chromosome 9 SNP rs4744411, and the *NLRP1* SNPs rs6502867 and rs4790797, we carried out CLRA in the combined case–pseudocontrol data set (generated by combining the case–pseudocontrol data sets of the two chromosome 7 SNPs, the one chromosome 9 SNP, and the two *NLRP1* SNPs derived from the combined 114 families) (Cordell and Clayton, 2002; Cordell *et al.*, 2004a) using a three-stage interaction testing framework (Millstein *et al.*, 2006) in order to test individual effects of signals represented by the five SNPs (stage 1), together with two-way interactions (stage 2) and with three-way interactions (stage 3). Analyses were carried out using STATA (http://www.stata.com), using the ''geneassoc'' routine (http://www-gene.cimr.cam.ac.uk/clayton/software/stata), and we assessed significance using a Wald test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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