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# Genome-Wide Association Study of Generalized Vitiligo in an Isolated European Founder Population Identifies *SMOC2*, in Close Proximity to *IDDM8*

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## Abstract

Generalized vitiligo is a common disorder in which patchy loss of skin and hair pigmentation principally appears to result from autoimmune loss of melanocytes from affected regions. We previously characterized a unique founder population in an isolated Romanian community with elevated prevalence of generalized vitiligo and other autoimmune diseases, including autoimmune thyroid disease, rheumatoid arthritis, and type I diabetes mellitus. Here, we describe a genome-wide association study (GWAS) of generalized vitiligo in 32 distantly related affected patients from this remote village and 50 healthy controls from surrounding villages. Vitiligo was significantly associated with single-nucleotide polymorphisms (SNPs) in a 30-kb LD block on chromosome 6q27, in close vicinity to *IDDM8*, a linkage and association signal for type I diabetes mellitus and rheumatoid arthritis. The region of association contains only one gene, *SMOC2*, within which SNP rs13208776 attained genome-wide significance for association with generalized vitiligo ($P = 8.51\times10^{-8}$) at odds ratio 7.445 (95% confidence interval=3.56–15.53) for the high-risk allele and population attributable risk 28.00. *SMOC2* encodes a modular extracellular calcium-binding glycoprotein of unknown function. Our findings indicate that *SMOC2* is a risk locus for generalized vitiligo and perhaps other autoimmune diseases.

## INTRODUCTION

Generalized vitiligo is the most common human pigmentation disorder, affecting approximately 0.4% of European Caucasians (CEU) (Howitz *et al*., 1977). Generalized vitiligo is characterized by acquired, progressive, multifocal patches of white skin and overlying hair that can result in significant social stigmatization, especially in patients from darker-skinned ethnic groups. Most evidence indicates that generalized vitiligo is an organ-specific autoimmune disease directed against melanocytes (Ongenae *et al*., 2003; Rezaei *et*



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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**SUPPLEMENTARY MATERIAL**

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

*al.*, 2007), and indeed about 20% of vitiligo patients (and their close relatives) manifest concomitant occurrence of other autoimmune diseases, particularly autoimmune thyroid disease, rheumatoid arthritis, late-onset type I diabetes mellitus, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease (Alkhateeb *et al.*, 2003). Nevertheless, heritable biological properties of the melanocyte or other factors, combined with environmental triggers, may contribute to loss of immune tolerance and ultimately autoimmunity directed against melanocytes (Boissy and Spritz, 2009).

Family clusters of vitiligo cases are not uncommon, occurring in a non-Mendelian pattern suggestive of polygenic, multifactorial inheritance (Spritz, 2007, 2008). Genetic linkage and association studies have implicated a number of genes in vitiligo pathogenesis, especially genes involved in immune function (Spritz, 2007, 2008). However, these loci account for a relatively small fraction of total disease liability.

Genetically isolated "founder populations" afford special opportunities to identify genes involved in susceptibility to disease, as founder populations may have elevated prevalence of specific diseases and reduced heterogeneity of causal genetic and environmental risk factors compared with more outbred populations (Wright *et al.*, 1999). Accordingly, susceptibility alleles that represent relatively minor genetic risk factors for complex diseases in the general population may become amplified and constitute major risk alleles in a founder population, and thus may be localized using less dense maps and smaller sample sizes than similar studies conducted in more outbred populations (Wittke-Thompson *et al.*, 2007). Although complex disease alleles detected as major signals in a unique genetic isolate may be problematic to replicate in the wider, outbred population, they nevertheless can represent important candidate genes that identify potential novel pathways of disease.

We have studied a geographically and genetically isolated community in a remote region of northern Romania, in which the prevalence of generalized vitiligo is 2.9%, at least eight times higher than in other European Caucasian groups (Birlea *et al.*, 2008). Similarly, the prevalence of other autoimmune diseases that are epidemiologically associated with vitiligo (autoimmune thyroid disease, rheumatoid arthritis, adultonset type I diabetes mellitus) are also elevated in this village, both in vitiligo patients and in their first-degree relatives. This village was founded approximately 400 years ago by only three families, and all affected individuals derive from a relatively small number of common ancestors (Birlea *et al.*, 2008); this village thus constitutes a unique founder population for generalized vitiligo.

We have taken advantage of the opportunity afforded by this special founder population to search for a generalized vitiligo susceptibility locus in this isolated village, in which reduced genetic heterogeneity enables testing a much smaller sample size than in a typical outbred population. We carried out a genome-wide association study (GWAS) of 310,598 SNPs in 32 villagers affected by generalized vitiligo from the Romanian founder population and 50 unrelated controls from surrounding villages. One SNP achieved genome-wide significance, rs13208776, located in *SMOC2* in distal chromosome 6q. Our results indicate that *SMOC2* is an important candidate susceptibility locus for generalized vitiligo in this isolated community, which may represent a microcosm of disease susceptibility in the broader CEU population.

## RESULTS AND DISCUSSION

We initially analyzed 32 of the most distantly related villagers affected with generalized vitiligo, carefully selected to minimize potential false association because of genetic relatedness, and 50 unrelated controls from immediately surrounding villages. All cases met strict clinical diagnostic criteria for generalized vitiligo (Taïeb and Picardo, 2007). Subject

DNAs were analyzed using Illumina Infinium Human Hap300 or CNV370 BeadChip microarrays, which interrogated 310,598 SNPs in common between the two platforms. We excluded two controls because of genotyping call rates <99%. In addition, we carried out genetic matching (Luca *et al.*, 2008) to control for population stratification by removing genetic outliers, thereby excluding four controls classified as outliers. We adjusted for relatedness among cases using CCREL software (Browning *et al.*, 2005), which estimated that the 32 distantly related cases corresponded to an effective sample size of 30.1 independent cases. After quality control procedures, we analyzed a final dataset that included 297,342 SNPs successfully genotyped in 32 distantly related cases and 44 unrelated controls.

Following genetic matching to minimize population stratification, we compared SNP allele frequencies in cases versus controls using Fisher single-marker exact tests implemented in PLINK, version 1.05 (Purcell *et al.*, 2007) and by allelic association tests that accounted for relatedness among cases using CCREL, version 0.3 (Browning *et al.*, 2005). Quantile–quantile (Q–Q) analyses of the observed $-\log_{10}(P\text{-values})$ from the Fisher exact tests (Figure 1a) and the observed $\chi^2$-test statistics from CCREL (Figure 1b) showed modest residual genomic inflation (Fisher's exact test genomic inflation factor $\lambda=1.06$; CCREL genomic inflation factor $\lambda=1.10$). Residual genomic inflation was effectively removed by adjustment of the test statistics for the corresponding inflation factor. After all corrections, we observed greater association than expected by chance (Table 1). As shown in Figure 2 and Supplementary Table S1, the most significant association signal was for SNP rs13208776 (nominal Fisher exact test *P*-value=$3.13\times10^{-8}$), which surpassed a strict Bonferroni-corrected criterion for genomewide significance ($P<1.68\times10^{-7}$; 0.05 divided by 297,342 SNPs; Ioannidis *et al.*, 2009), based on the *P*-values from both the Fisher exact test (adjusted $P=8.51\times10^{-8}$) and from the CCREL likelihood ratio $\chi^2$ test (adjusted $P=9.71\times10^{-8}$). The adjusted Fisher exact *P*-value for rs13208776 exceeded the 2.1% quantile of minimum *P*-values from 1,000 permutations of the phenotype labels, and was the only *P*-value outside the 95% confidence limits that were estimated empirically for each of the 20 top-ranking GWAS signals. Together, these analyses show that rs13208776 surpasses a conservative Bonferroni-corrected significance threshold, even after adjustment for relatedness among cases and for genomic inflation.

SNP rs13208776 is located on chromosome 6q27, within intron 4 of the *SMOC2* gene (Figure 3). Of the 10 SNPs showing the strongest association within the *SMOC2* region, most were contained in two adjacent linkage disequilibrium blocks, the first comprising seven SNPs (average $D'=0.97$; $r^2=0.40$), including rs13208776, and the second comprising two SNPs ($D'=1.00$; $r^2=0.04$), rs1402|rs214479; SNP rs2144749 had an adjusted *P*-value $1.75\times10^{-4}$ and is substantially correlated ($D'=0.72$; $r^2=0.49$) with SNP rs13208776. We identified several haplotypes in this region with *P*-values slightly improved over SNP rs13208776, although none were significantly better. Of 297,342 SNPs tested, the 20 SNPs with the most extreme *P*-values associated with vitiligo are listed in Supplementary Table S1.

*SMOC2*, which is the only gene in this region of 6q27, is located in close proximity to *IDDM8* (http://www.t1dbase.org), a genetic locus that has both linkage and association with type I diabetes (Luo *et al.*, 1995; Davies *et al.*, 1996; Owerbach, 2000; Cox *et al.*, 2001) and rheumatoid arthritis (Myerscough *et al.*, 2000). Type I diabetes and rheumatoid arthritis are autoimmune diseases that are epidemiologically associated with generalized vitiligo, both in the outbred CEU population in general (Alkhateeb *et al.*, 2003) and in this Romanian founder population specifically (Birlea *et al.*, 2008). Recently, SNPs within intervening sequence 4 of *SMOC2* have been genetically associated with measures of pulmonary function (Wilk *et al.*, 2007), although none of these associations remain significant after

multiple-testing correction. Our findings indicate that *SMOC2* is an important novel candidate gene for susceptibility to generalized vitiligo, and perhaps to other autoimmune diseases, in the isolated Romanian founder population of this study. This finding may be difficult to verify in the broader CEU population, as genetic purification in this unique population isolate has led to a very high population attributable risk for this *SMOC2* susceptibility allele that may be much smaller in the outbred CEU population, even for the same variant. *SMOC2* encodes a widely expressed, SPARC (BM40)-related glycoprotein that contains two thyroglobulin type-I domains, two EF-hand calcium-binding domains, a follistatin-like domain, and a putative signal peptide (Nishimoto *et al.*, 2002; Vannahame *et al.*, 2003). The specific function of the SMOC-2 protein remains unknown, though roles have been suggested in angiogenesis (Rocnik *et al.*, 2006), cell cycle regulation (Liu *et al.*, 2009) and mitogenesis (Liu *et al.*, 2009). Although defective calcium transport has been reported in vitiligo melanocytes and keratinocytes (Schallreuter-Wood *et al.*, 1996), specific involvement of SMOC-2 in calcium homeostasis remains unproven.

In the skin, SMOC-2 is mainly present in the basal levels of the epidermis, and SMOC-2-stimulated attachment of primary keratinocytes in culture (Maier *et al.*, 2008). Together, these findings are of interest in light of the suggestion that melanocyte loss in vitiligo might result from chronic cell detachment due to defective cell adhesion (Gauthier *et al.*, 2003). Nevertheless, it is not apparent how this etiopathological mechanism might also result in the frequent concomitant occurrence of other autoimmune diseases in vitiligo patients from this isolated community, such as autoimmune thyroid disease, type I diabetes, and rheumatoid arthritis.

## MATERIALS AND METHODS

### Subjects

The genealogy and demographic characteristics of the Romanian study community, which comprises 1673 Caucasian individuals (2004 census), have been described previously (Birlea *et al.*, 2008). Genealogical relationships were known as early as the mid-16th century, when the community was founded by three families. The population has remained substantially isolated, with a low rate of immigration; most marriages are between neighbors and involve distant consanguinity. All study participants were examined clinically, using standard diagnostic criteria for vitiligo (Taïeb and Picardo, 2007), and were interviewed regarding ancestry.

We identified 59 living and deceased villagers with vitiligo, all of whom could be linked to one large pedigree (Birlea *et al.*, 2008), and selected the 32 most distantly related community inhabitants with generalized vitiligo (22 females, 10 males; cousins of fourth and higher degrees) for this study. Fourteen patients had other autoimmune disorders, including autoimmune thyroid disease (10 patients), type I diabetes (4 patients), and rheumatoid arthritis (3 patients). In addition, we selected as controls 50 unrelated adult individuals (42 female and 8 male patients), without vitiligo or other autoimmune diseases and with no known ancestral ties to the founder community, collected from immediately surrounding villages in which the prevalence of vitiligo is very low, ~0.15%. Informed consent was obtained from all subjects and the study was conducted according to the Declaration of Helsinki Principles. The study was approved by the Ethics Committee of Iuliu Hatieganu University of Cluj-Napoca, Romania and by COMIRB at the University of Colorado Denver.

### DNA preparation, genome-wide genotyping, and data quality control

DNA was isolated from peripheral blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin) and DNA concentrations were quantified with PicoGreen (Molecular Probes, Eugene, Oregon). Genome-wide genotyping was carried out at Decode Genetics (Reykjavik, Iceland) using 3 μg DNA and Illumina Infinium Human Hap300 or CNV370 microarrays, with 310,598 SNPs common to both platforms and hence considered here. Genotypes were determined using Illumina Bead Studio software.

SNP genotype quality control analyses were carried out using the PLINK tool set (version 1.05) (Purcell *et al.*, 2007). A total 13,256 SNPs were excluded because of call rates <0.95 ($n$=483), minor allele frequency <0.05 (Liu *et al.*, 2008) ($n$=12,761), SNPs that mapped to both X and Y chromosomes ($n$=2), and SNPs that deviated significantly ($P<10^{-4}$) from Hardy–Weinberg in the control population ($n$=10). Two controls were excluded because of overall SNP call rate <99%. Genetic matching and outlier exclusion was carried out using principal component analysis implemented in genetic matching software (Luca *et al.*, 2008) to a correlation matrix of 13,762 independent SNPs ($r^2<0.09$) identified using the "indep-pairwise" pruning option in PLINK. Four control individuals with ancestry coefficients exceeding six SD were excluded.

### Statistical analyses

Fisher exact tests of single marker and haplotype association were carried out using PLINK, version 1.05 (Purcell *et al.*, 2007); allelic association tests that accounted for relatedness among cases were carried out using CCREL, version 0.3 (Browning *et al.*, 2005). Haplotype frequencies were estimated using PHASE, version 2.1 (Stephens and Donnelly, 2003), and LD blocks were delineated using HAPLOVIEW, version 3.32 (Barrett *et al.*, 2005). A quantile—quantile (Q–Q) plot of observed and expected $-\log_{10}(P\text{-values})$ from Fisher exact tests (Figure 1a), with expected values derived as $P=r/(n+1)$, in which $r$ corresponds to the rank of $n$=297,342 ordered $P$-values, was generated and the inflation of the observed $-\log_{10}(P\text{-values})$ was measured as the slope of the best-fitting line using R software, version 2.9.0 (http://www.r-project.org/). 95% confidence limits for the 20 top-ranked $-\log_{10}(P\text{-values})$ on the plot were estimated from 1,000 permutations of the phenotype labels. A Q–Q plot corresponding to the observed *versus* expected $\chi^2$-statistics from the CCREL analysis (Figure 1b) was generated and the genomic inflation factor for the observed $\chi^2$-statistics was estimated as the observed median $\chi^2$ divided by the expected median $\chi^2$ (Devlin *et al.*, 2001).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **CEU** | non-Hispanic Caucasians of European origin |
| **GWAS** | genome-wide association study |

**SNP** single-nucleotide polymorphism

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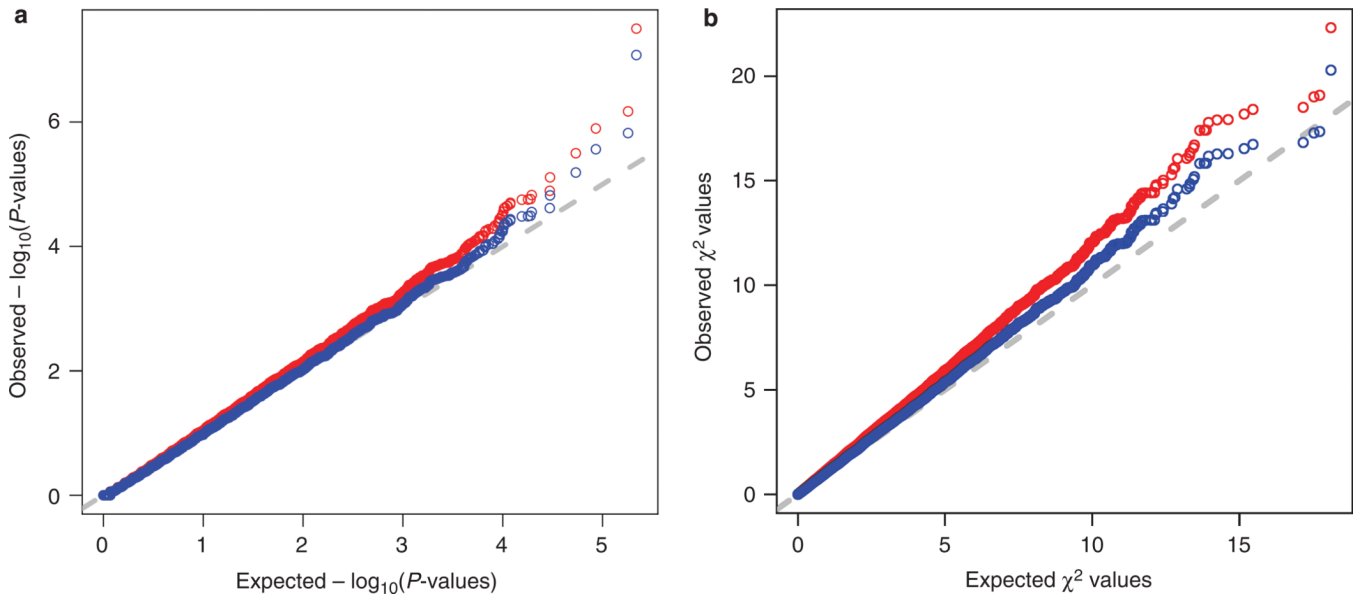


**Figure 1. Quantile–quantile plots of observed and expected test statistics**

(**a**) Comparison of observed $-\log_{10}$(*P*-values) from Fisher exact tests to those expected for a null distribution. Plots are shown before (red dots) and after (blue dots) adjustment of *P*-values for the corresponding genomic inflation factor (λ) 1.06. (**b**) Comparison of observed CCREL (Browning *et al.*, 2005) $\chi^2$-values to those expected for a null distribution. Plots are shown before (red dots) and after (blue dots) adjustment of $\chi^2$-statistics for the corresponding inflation factor (λ) 1.10 using the genomic control method (Devlin *et al.*, 2001).

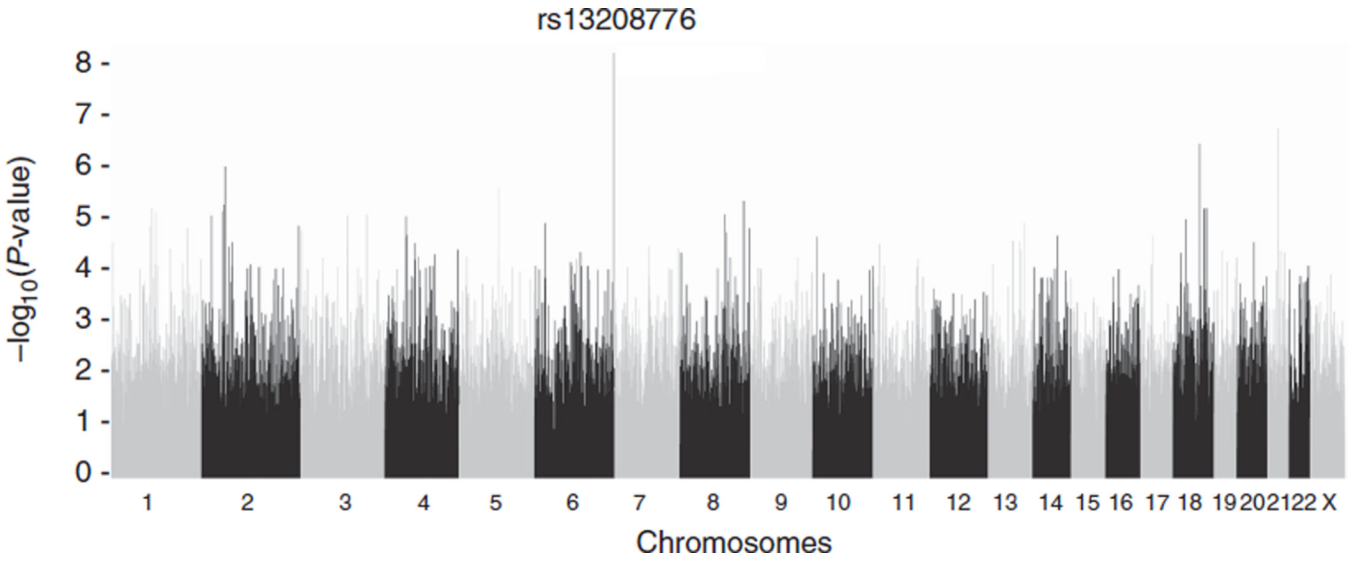


**Figure 2. SNP $-\log_{10}$(*P*-values) for the Fisher exact test statistics from the GWAS plotted according to chromosomal position**

The genome-wide distribution of $-\log_{10}$(*P*-values) for the comparison of single-nucleotide polymorphism (SNP) allele frequencies in generalized vitiligo cases versus unrelated controls by the Fisher exact test of allele frequencies for 297,342 SNPs that passed quality control filters. *P*-values were corrected for the corresponding genomic inflation factor (λ) 1.06. SNP nucleotide positions are based on National Center for Biotechnology Information Build 36.1.

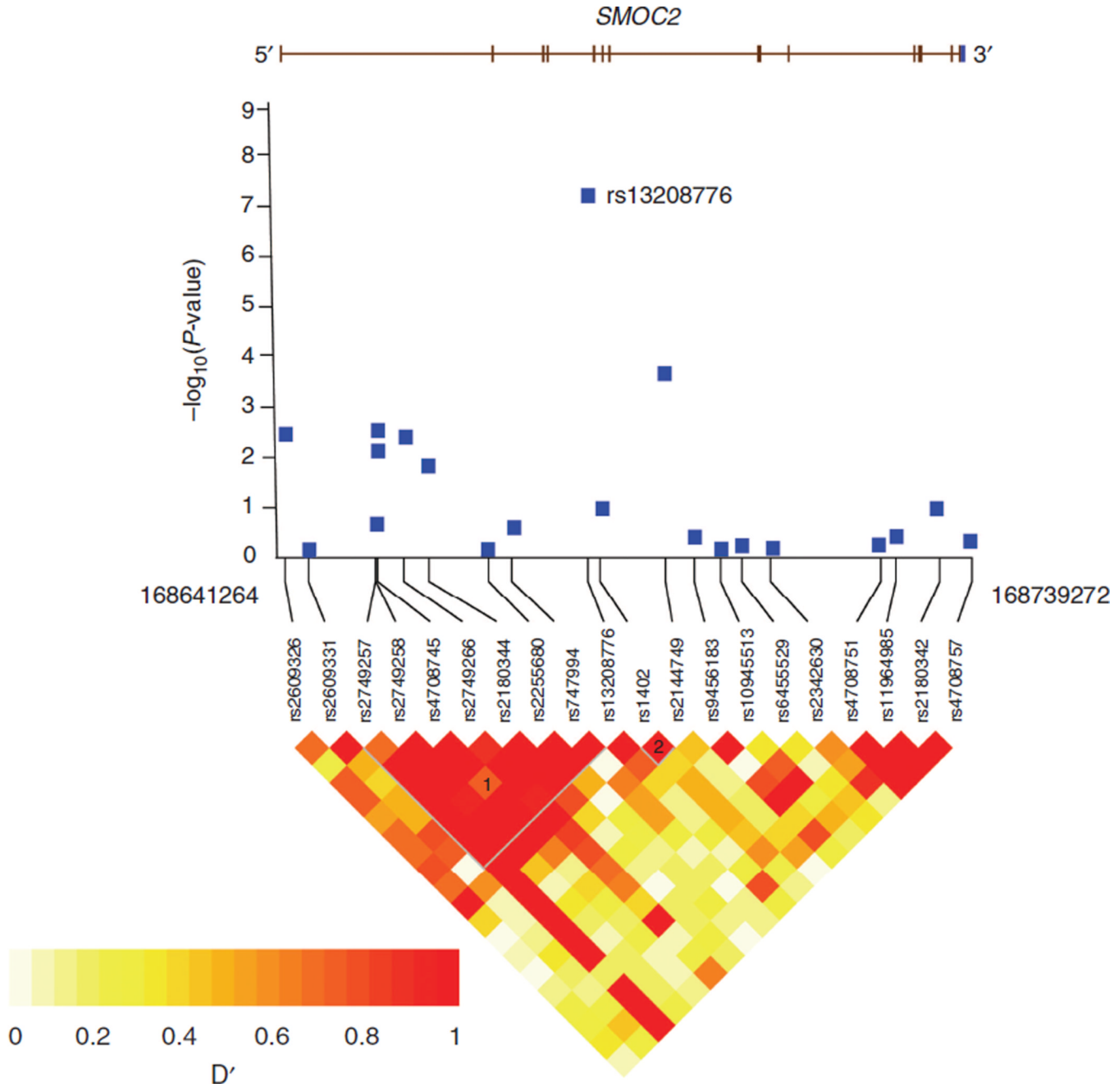


**Figure 3. Schematic view of association in the *SMOC2* region of chromosome 6q27**

The top panel shows −$\log_{10}$(*P*-values) from Fisher exact tests comparing allele frequencies in cases versus controls for 20 single-nucleotide polymorphisms (SNPs) located in the *SMOC2* region. *P*-values were adjusted for the corresponding genomic inflation factor (λ) 1.06. The middle panel shows SNP rs numbers and nucleotide positions (National Center for Biotechnology Information Build 36.1). The bottom panel shows a Haploview (Barrett *et al.*, 2005) representation of linkage disequilibrium (D′) based on genotyping data for these SNPs from the Romanian case–control group.

## Table 1

Number of significant associations identified in GWAS of Romanian population isolate for generalized vitiligo

| Level of significance | Observed | Observed adjusted[1] | Expected | Ratio |
|---|---|---|---|---|
| 0.01–0.05 | 15,998 | 13,548 | 11,894 | 1.13 |
| 0.001–0.01 | 4,536 | 3,299 | 2,676 | 1.23 |
| 0.0001–0.001 | 591 | 365 | 268 | 1.36 |
| 0.00001–0.0001 | 74 | 30 | 27 | 1.11 |
| <0.00001 | 5 | 4 | 2 | 2.00 |
| All $P$<0.05 | 21,204 | 17,246 | 14,867 | 1.16 |

Observed numbers of SNPs associated with generalized vitiligo, by level of significance, before and after genomic control adjustment for population stratification, and expected number under the null hypothesis of no association.

[1]Adjusted for inflation of test statistic by the genomic control method (Devlin *et al.*, 2001).