ARTHRITIS & RHEUMATISM
Vol. 63, No. 11, November 2011, pp 3607–3612
DOI 10.1002/art.30604

A Putative Functional Variant Within the *UBAC2* Gene Is Associated With Increased Risk of Behçet's Disease

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Objective. **Using a genome-wide association scan and DNA pooling, we previously identified 5 novel genetic susceptibility loci for Behçet's disease. We undertook this study to establish the genetic effect within the *UBAC2* gene, in the course of which we replicated this genetic association and identified a functional variant within this locus.**

Methods. **We studied a total of 676 Behçet's disease patients and 1,096 controls. The discovery set included 156 patients and 167 controls from Turkey, and the replication sets included 376 patients and 369 controls from Turkey and 144 patients and 560 controls from Italy. Genotyping of 14 single-nucleotide polymorphisms (SNPs) within and around *UBAC2* was performed using TaqMan SNP genotyping assays.**

Results. **The genetic association between Behçet's disease and *UBAC2* was established, replicated, and confirmed (meta-analysis odds ratio 1.84, $P = 1.69 \times 10^{-7}$). Haplotype analysis identified both a disease-risk haplotype and a protective haplotype ($P = 0.00014$ and $P = 0.0075$, respectively). Using conditional haplotype analysis, we identified the SNP rs7999348 (A/G) within *UBAC2* as the most likely SNP with a genetic effect independent of the haplotypic effect formed by the remaining associated SNPs in this locus. Indeed, we demonstrated that rs7999348 tags a functional variant associated with increased messenger RNA expression of a *UBAC2* transcript variant in peripheral blood mononuclear cells of individuals homozygous for the Behçet's disease–associated "G" allele. Further, our data suggested the possibility of multiple genetic effects that increase susceptibility to Behçet's disease in the *UBAC2* locus.**

Conclusion. **We established and confirmed the genetic association between *UBAC2* and Behçet's disease in 3 independent sets of patients and controls. We identified the minor allele in rs7999348 as a disease-risk allele that tags altered *UBAC2* expression.**

Behçet's disease is a systemic inflammatory disease characterized by the presence of recurrent orogenital ulceration, inflammatory eye disease, central nervous system involvement, skin involvement, and gastrointestinal involvement. Other disease manifestations include arthritis, arterial aneurysms, and recurrent deep venous thrombosis. The disease is most common along the ancient "silk road" route, and thus is most prevalent in East Asia, the Middle East, North Africa, and southern Europe. Both men and women are equally affected; however, younger patients and men tend to have a more severe disease with higher morbidity and mortality (1).

The pathogenesis of Behçet's disease is poorly understood. Evidence for a genetic contribution to the disease etiology is largely derived from familial aggrega-

Supported by the American College of Rheumatology Research and Education Foundation (Rheumatology Investigator Award to Dr. Sawalha) and by the NIH (grants R03-AI-076729, P20-RR-020143, and P30-AR-053483).

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Submitted for publication January 18, 2011; accepted in revised form August 2, 2011.

tion of the disease (2), disease incidence studies in immigrant populations (3), and the universally confirmed association between Behçet's disease and HLA–B51, which is estimated to account for ~19% of the genetic risk for this disease (4). Evidence for involvement of environmental factors includes the association of poor oral health with Behçet's disease (5,6). Evidence for possible infectious etiology contributing to Behçet's disease comes from the high frequency of isolating *Staphylococcus aureus* from acne lesions in Behçet's disease patients compared to acne vulgaris patients (7), the presence of higher titers of anti–mycobacterial heat-shock protein antibodies in patients' sera (8), and the more frequent mouth colonization with *Streptococcus mutans* in patients compared to controls (9).

Recently, we performed a genome-wide association study (GWAS) in a set of Turkish Behçet's disease patients and controls, and identified 5 novel candidate genetic susceptibility loci for the disease (10). These candidate loci included *KIAA1529*, *CPVL*, *LOC100129342*, *UBASH3B*, and *UBAC2*. Two subsequent GWAS in Behçet's disease established and validated the genetic association between *IL10* and *IL23R* and Behçet's disease (11,12).

In this study, we genotyped 14 single-nucleotide polymorphisms (SNPs) in the *UBAC2* locus and identified a functional tag SNP within *UBAC2* that increases susceptibility to Behçet's disease. Further, we confirmed and replicated the genetic association in the *UBAC2* genetic locus in a total of 3 independent sets of patients and controls.

PATIENTS AND METHODS

Patients and controls. Three independent sets of Behçet's disease patients and ethnically matched controls from Turkey and Italy were included in this study. The first set included 156 patients and 167 controls from Turkey, the second set included 376 patients and 369 controls from Turkey, and the third set included 144 patients and 560 controls from Italy. All patients fulfilled the 1990 International Study Group classification criteria for Behçet's disease (13). The study protocols were approved by the ethics committees and Institutional Review Boards at our institutions. All study participants signed an informed written consent. Buffy coat samples from normal blood donors were obtained from the Oklahoma Blood Institute and used to separate peripheral blood mononuclear cells (PBMCs) to measure *UBAC2* transcript levels.

Genotyping and data analysis. Genotyping of SNPs within and around the *UBAC2* gene was performed using TaqMan SNP Genotyping Assays (Applied Biosystems). A total of 14 SNPs were genotyped in this study. The SNPs selected for genotyping represent common genetic variants within the *UBAC2* locus that showed a genetic association with Behçet's disease in our pooled DNA GWAS. Our GWAS included 59 SNPs located in the linkage disequilibrium (LD) block containing *UBAC2* (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). Forty-eight of these SNPs, also included in HapMap, capture 86% of variants in this LD block with a mean r^2 value of 0.978 in the population of Utah residents with Northern and Western European ancestry combined with the population of Toscani residents in Italy. Only individuals with a genotyping success rate of >90% were used for subsequent analysis. All SNPs had a genotyping success rate of >90%.

Allele frequencies in patients and controls were determined. A chi-square test was used to examine genetic association between each of the genotyped SNPs and Behçet's disease, and odds ratios (ORs) and 95% confidence intervals (95% CIs) were determined. Hardy-Weinberg equilibrium *P* values were calculated in controls using Haploview 4.2 (14) and were not significant (>0.05). Haplotype analysis was performed using Haploview 4.2 and PLINK (14,15). Conditional haplotype analysis and meta-analysis were performed using PLINK (15). Each genotyped SNP was individually examined for an independent genetic effect against the haplotypic background formed by all remaining SNPs.

PBMC separation, RNA extraction, and real-time reverse transcription–polymerase chain reaction (RT-PCR). Using density-gradient centrifugation (Amersham Biosciences), PBMCs were separated from buffy coat samples obtained from normal healthy blood donors. DNA was extracted using the DNeasy Kit (Qiagen), and RNA was extracted using a combination of TRIzol (Invitrogen) and the RNeasy kit

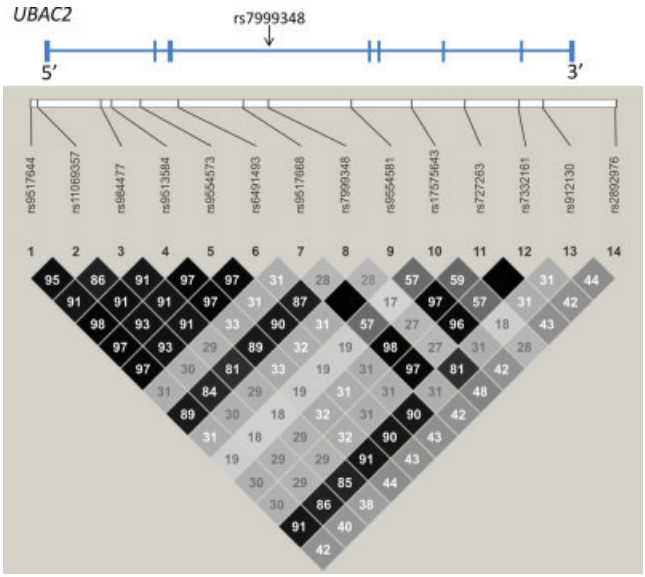

Figure 1. Linkage disequilibrium plot showing the 14 single-nucleotide polymorphisms (SNPs) genotyped in the *UBAC2* locus and pairwise correlation coefficient (r^2) values. **Arrow** indicates location of the newly characterized functional SNP rs7999348.

Table 1. Genetic association between *UBAC2* and Behçet's disease*

SNP	Associated allele	Frequency		OR (95% CI)	*P*
		Patients	Controls		
rs9517644	T	0.44	0.32	1.72 (1.23–2.41)	0.0013
rs11069357	A	0.45	0.33	1.68 (1.21–2.35)	0.002
rs984477	G	0.46	0.34	1.65 (1.17–2.32)	0.0043
rs9513584	G	0.45	0.32	1.74 (1.23–2.46)	0.0017
rs9554573	A	0.44	0.32	1.73 (1.24–2.43)	0.0012
rs6491493	G	0.44	0.32	1.74 (1.25–2.43)	0.0011
rs9517668	T	0.23	0.10	2.62 (1.64–4.18)	3.61×10^{-5}
rs7999348	G	0.48	0.34	1.78 (1.28–2.48)	0.00058
rs9554581	T	0.22	0.10	2.48 (1.56–3.95)	8.53×10^{-5}
rs17575643	T	0.15	0.06	2.91 (1.63–5.20)	0.00018
rs727263	A	0.23	0.11	2.45 (1.55–3.88)	1.00×10^{-4}
rs7332161	A	0.22	0.11	2.43 (1.54–3.85)	0.00011
rs912130	G	0.44	0.33	1.58 (1.13–2.21)	0.0071
rs2892976	G	0.36	0.23	1.96 (1.37–2.80)	0.00023

* Fourteen single-nucleotide polymorphisms (SNPs) in the *UBAC2* genetic locus were genotyped in a set of 156 patients and 167 normal healthy controls. OR = odds ratio; 95% CI = 95% confidence interval.

(Qiagen), as previously described (16). RNA was treated with Turbo DNA-free (Ambion) to digest any contaminating DNA. Real-time RT-PCR was performed to measure the relative concentration of *UBAC2* transcript variants 1 and 2, with normalization to the housekeeping gene β-actin, using the iScript One-Step RT-PCR Kit With SYBR Green (Bio-Rad) and the Rotor-Gene 3000 real-time thermocycler (Corbett Research). The PCR steps used were as follows: 50°C for 10 minutes, 95°C for 5 minutes, and 45 cycles of 95°C for 10 seconds followed by 55°C for 30 seconds. Primer sequences were as follows: for *UBAC2* transcript variant 1, forward 5′-GCTCCAGTGGGCTCTACAAG-3′ and reverse 5′-CCTCCAAATCTGGAAGTCGT-3′; for *UBAC2* transcript variant 2, forward 5′-TGCTGGATGTTGCTGTTTTC-3′ and reverse 5′-CAGGCTGGAAGTCGTTCTTG-3′; for β-actin, forward 5′-GCACCACACCTTCTACAATGAGC-3′ and reverse 5′-GGATAGCACAGCCTGGATAGCAAC-3′.

RESULTS

We have previously reported the candidate association between *UBAC2* and Behçet's disease. This association was initially discovered in our GWAS using pooled DNA samples from Behçet's disease patients and controls, and was later validated with single-sample genotyping in the same set ($P = 5.8 \times 10^{-3}$) (10). Here we genotyped 14 SNPs within and around *UBAC2* (Figure 1). These experiments were performed using the 156 patients and 167 controls included in our discovery set. The SNPs selected for genotyping were SNPs that showed evidence for genetic association with Behçet's disease in our GWAS using pooled DNA samples in the *UBAC2* locus (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

We found genetic association between all 14 SNPs genotyped in the *UBAC2* locus and Behçet's disease (Table 1). The SNP rs9517668 located in the third intron within *UBAC2* showed the most significant association among the genetic variants tested (OR 2.62, $P = 3.61 \times 10^{-5}$).

The association between rs9517668 within *UBAC2* and Behçet's disease was then replicated and confirmed in a second larger independent set consisting of 376 patients and 369 controls from Turkey (OR 1.82 [95% CI 1.31–2.53], $P = 0.00034$). This association was not detected in the Italian set of Behçet's disease patients and controls (OR 1.41 [95% CI 0.91–2.18], $P =$

Table 2. Meta-analysis for Behçet's disease–associated alleles in rs9517668 and rs7999348 within the *UBAC2* gene in 3 independent sets of Behçet's disease patients and controls*

	SNP (associated allele)	
	rs9517668 (T)	rs7999348 (G)
Turkish set 1		
OR	2.62	1.78
P	3.61×10^{-5}	0.00058
Turkish set 2		
OR	1.82	1.29
P	0.00034	0.023
Italian set		
OR	1.41	1.40
P	0.12	0.018
Meta-analysis		
OR	1.84	1.39
P	1.69×10^{-7}	1.85×10^{-5}
P, heterogeneity	0.21	0.49

* SNP = single-nucleotide polymorphism; OR = odds ratio.

Table 3. Haplotype analysis using 14 SNPs genotyped in the *UBAC2* locus reveals both a disease-risk haplotype and a protective haplotype in Behçet's disease.

Haplotype	Frequency		*P*
	Patients	Controls	
CGCAGCAACCGGTA	0.50	0.62	0.0075
TAGGAGAGCCGGGG	0.10	0.09	0.52
TAGGAGTGTTAAGG	0.16	0.06	0.00014
TAGGAGAGCCGGGA	0.11	0.12	0.6

* The order of single-nucleotide polymorphisms (SNPs) within the haplotypes presented (from left to right) is maintained as presented in Figure 1 (from left to right) and in Table 1 (from top to bottom). Only haplotypes with frequencies of at least 5% are depicted.

0.12). However, a meta-analysis for the association between rs9517668 and Behçet's disease in the 3 independent sets included in this study revealed a meta-analysis OR of 1.84 and a *P* value of 1.69×10^{-7} (Table 2). Haplotype analysis using all 14 SNPs genotyped in the discovery set identified 4 common haplotypes (frequency ≥5%), including a disease-risk haplotype with frequencies of 16% in patients and 6% in controls ($P = 0.00014$) and a protective haplotype with frequencies of 50% in patients and 62% in controls ($P = 0.0075$) (Table 3).

Conditional haplotype analysis identified rs7999348 as the most likely SNP of the 14 genotyped SNPs that might have an independent genetic effect against the haplotypic effect formed by the remaining associated SNPs in this locus (likelihood ratio 3.25, $P = 0.071$) (see Supplementary Table 2, available on the *Arthritis & Rheumatism* Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131). Genotype model association tests in rs7999348 suggested an additive model for this genetic effect, as the Cochran-Armitage test *P* value (0.00078) was lower than the *P* values for the dominant or recessive models (0.0059 and 0.0053, respectively). The genetic association between rs7999348 and Behçet's disease was replicated in the second independent set of Turkish patients and controls (OR 1.29 [95% CI 1.04–1.60], $P = 0.023$) and also in the Italian patients and controls (OR 1.40 [95% CI 1.06–1.86], $P = 0.018$). Table 2 presents the results of a meta-analysis for the association of rs7999348 with Behçet's disease in our 3 independent sets.

The SNP rs7999348 is an intronal SNP located within the *UBAC2* gene. Using SNP function prediction algorithms incorporated within the FASTSNP software (17), it is predicted that rs7999348 changes transcription factor binding, with a small predicted probability of altering an intronic enhancer. Therefore, we hypothesized that disease-causing variants within the *UBAC2* gene might alter the expression levels of *UBAC2* transcripts. We tested messenger RNA (mRNA) expression of the 2 known protein-coding *UBAC2* transcript variants in PBMCs obtained from normal healthy donors who carry the homozygous risk genotype (GG), the homozygous protective genotype (AA), and the heterozygous genotype (AG) in rs7999348. We found that the expression of *UBAC2* transcript variant 1 (NM_001144072.1) was significantly increased in the presence of the homozygous risk genotype in rs7999348 compared to both the heterozygous genotype and the homozygous protective genotype in this SNP (mean ± SEM relative mRNA expression 2.79 ± 0.63 [n = 4], 0.91 ± 0.12 [n = 8], and 0.69 ± 0.19 [n = 8] in GG, AG, and AA, respectively [$F = 14.23$, $P = 0.0002$ by one-way analysis of variance]) (Figure 2). Using either parametric (*t*-test) or nonparametric (Mann-Whitney test) analysis, there was a significant difference in *UBAC2* transcript variant 1 expression in individuals with the homozygous risk genotype compared to individuals with the homozygous protective genotype or individuals with the heterozygous genotype (for GG versus AA, $P = 0.002$ by *t*-test, $P = 0.004$ by Mann-Whitney test; for GG versus AG, $P = 0.0021$ by *t*-test, $P = 0.004$ by Mann-Whitney test). We did not find a significant difference in the expression of *UBAC2* transcript variant 2 (NM_177967.3) between the various genotypes (mean ± SEM relative mRNA expression 0.95 ± 0.28 [n = 4], 1.24 ± 0.81 [n =

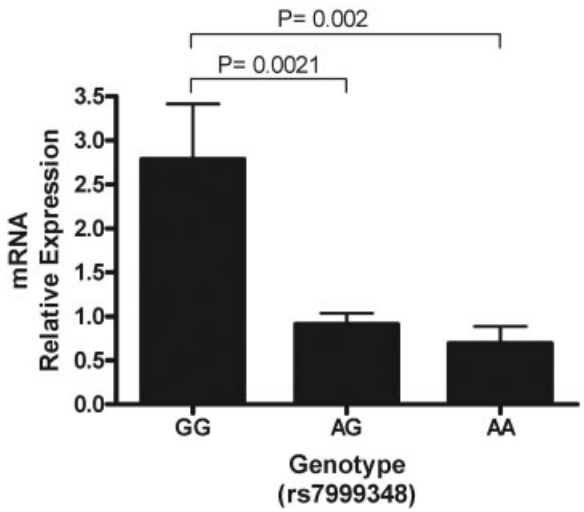

Figure 2. Relative mRNA expression of *UBAC2* transcript variant 1 (NM_001144072.1) in peripheral blood mononuclear cells obtained from normal healthy donors who carry the homozygous risk genotype (GG) (n = 4), the heterozygous genotype (AG) (n = 8), and the homozygous protective genotype (AA) (n = 8) in rs7999348. Values are the mean ± SEM.

8], and 2.68 ± 0.94 [n = 8] in GG, AG, and AA, respectively [F = 1.13, $P = 0.35$ by one-way analysis of variance]). Transcript variant 2, which is shorter than transcript variant 1, is missing 2 consecutive in-frame coding exons and contains an alternate 5′-end exon leading to a different N-terminus compared to transcript variant 1.

To validate allele-specific *UBAC2* expression in rs7999348, we used the Gene Expression Variation (GENEVAR) expression quantitative trait loci database (18). While rs7999348 was not included in this database, we found a SNP that is in strong LD with rs7999348 (rs2181502; $r^2 = 0.88$ with rs7999348). This SNP shows an allele-specific gene expression profile similar to our findings. The minor allele in rs2181502 (which tags the minor and Behçet's disease–associated allele [G] in rs7999348) is associated with significant overexpression of *UBAC2* in lymphoblastoid cell lines ($P = 0.03$) (see Supplementary Figure 1, available on the *Arthritis & Rheumatism* Web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

The most significant genetic effect detected in the *UBAC2* locus was with the SNP rs9517668 located within *UBAC2* ($P = 1.69 \times 10^{-7}$). There was relatively low LD between rs9517668 and rs7999348 ($r^2 = 0.28$). This suggested that the genetic effect in rs9517668 might be independent of rs7999348. Indeed, a 2-SNP haplotype regression analysis revealed that the haplotypic genetic association *P* value for the 2-SNP haplotype formed by rs9517668 and rs7999348 (0.00055) maintained significance independent of rs7999348 ($P = 0.012$). This highlights the possibility that rs9517668 tags a genetic effect that increases the susceptibility to Behçet's disease independent of rs7999348, and that multiple independent genetic effects for Behçet's disease might be present in this locus and will need to be further investigated.

DISCUSSION

Behçet's disease is a multisystem inflammatory disease of unclear etiology. The repeatedly confirmed genetic association between the disease and the HLA locus leaves no doubt that genetic factors play an important role in the pathogenesis of the disease. Despite being the only confirmed genetic association in all ethnicities, there is no established mechanistic explanation to date for how HLA–B51 increases the susceptibility for developing Behçet's disease. Further, a lack of association has been observed between disease severity and HLA–B51 (19).

It became apparent in Behçet's disease, as in a number of other rheumatic and inflammatory diseases, that the association with the HLA region did not fully account for the disease genetic risk. Indeed, GWAS in the last few years have provided a large number of candidate genes for various immune-mediated diseases; some highlighted novel therapeutic targets, such as the association with *IL23R* in inflammatory bowel disease (20).

We previously performed the first GWAS in Behçet's disease and identified at least 5 novel candidate susceptibility loci for the disease outside of the HLA region (10). We also confirmed the known association with HLA–B51 in our sample set (Saruhan-Direskeneli G, et al: unpublished observations).

In the present study, we have established the genetic association in the *UBAC2* locus and confirmed this association in 2 additional sets of patients and controls representing 2 ethnic groups. Using conditional haplotype analysis, we found that rs7999348 was the most likely SNP among the genotyped SNPs to have a genetic effect independent of the haplotypic effect formed by the remaining associated SNPs in this locus. Indeed, we found that the expression of a *UBAC2* transcript variant was significantly increased in individuals with the Behçet's disease–associated homozygous genotype in this variant compared to heterozygotes ($P = 0.0021$) and compared to individuals with the protective genotype ($P = 0.002$). These findings are also consistent with *UBAC2* expression profiles in the GENEVAR expression quantitative trait loci database. Further, our data suggest that rs9517668 increases the susceptibility to Behçet's disease independent of rs7999348, raising the possibility of multiple genetic effects in the *UBAC2* locus. Resequencing will be needed to identify disease-causal variants in this locus. Future efforts should also focus on determining the effect of altered *UBAC2* expression on the pathogenesis of Behçet's disease. While our data support the genetic association between *UBAC2* and Behçet's disease in 2 Caucasian populations, a similar finding in other ethnic groups remains to be determined.

The function of the protein encoded by *UBAC2* is not known. However, the presence of a ubiquitin-associated domain in this gene product predicts involvement in ubiquitination pathways. Indeed, *UBAC2* mRNA is ubiquitously expressed (BioGPS database). Of interest, we have previously reported the genetic association of Behçet's disease with another ubiquitination-related gene (*UBASH3B*) (10). The genetic association of Behçet's disease with a third ubiquitination-related

gene (*SUMO4*) has also been reported (21). These data suggest that ubiquitination defects might be involved in the pathogenesis of Behçet's disease.

In summary, we establish and replicate a genetic association between Behçet's disease and the *UBAC2* gene. Indeed, we identify a functional variant–tagging SNP that increases the risk of Behçet's disease and that is associated with higher mRNA expression of a common *UBAC2* splice variant in PBMCs.

ACKNOWLEDGMENTS

We would like to thank Dr. Sandra D'Alfonso, Dr. Raffaella Scorza, Dr. Angela Tincani, and Dr. Andrea Doria for providing control samples for our study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sawalha had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sawalha, Hughes, Direskeneli.

Acquisition of data. Sawalha, Hughes, Nadig, Yılmaz, Aksu, Keser, Cefle, Yazıcı, Ergen, Alarcón-Riquelme, Salvarani, Casali, Direskeneli, Saruhan-Direskeneli.

Analysis and interpretation of data. Sawalha, Hughes, Saruhan-Direskeneli.

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