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***CTLA4* and generalized vitiligo: two genetic association studies and a meta-analysis of published data**

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Summary

Several lines of evidence have implicated the gene encoding cytotoxic T lymphocyte antigen 4 (*CTLA4*) in susceptibility to various autoimmune diseases. However, published studies of genetic association between *CTLA4* polymorphisms and vitiligo have yielded conflicting results. Here, we describe two new genetic association studies of *CTLA4* single-nucleotide polymorphisms (SNPs) and generalized vitiligo in two independent Romanian Caucasian (CEU) case-control cohorts. The first study, of SNPs rs1863800, rs231806, rs231775, rs3087243, rs11571302, rs11571297, and rs10932037, showed no allelic, genotypic, or haplotypic association with generalized vitiligo. The second study, of SNP rs231775, likewise showed no significant association. To enhance statistical power over that of any individual study, we carried out a meta-analysis that incorporated these two new studies and all other published genetic association studies of *CTLA4* SNPs and vitiligo in CEU populations. While there was no association with vitiligo overall, the meta-analysis showed significant association of SNP rs231775 in that subgroup of vitiligo patients who also had other concomitant autoimmune diseases. Similarly, there was near-significant association in this same patient subgroup with several other *CTLA4* SNPs that are in linkage disequilibrium with rs231775. Our results indicate that the association of *CTLA4* with vitiligo is weak, and indeed may be secondary, driven by primary genetic association of *CTLA4* with other autoimmune diseases that are epidemiologically associated with vitiligo.

Keywords

CTLA4; Vitiligo; genetic association; SNP; meta-analysis

Introduction

Generalized vitiligo is a disorder in which acquired patchy skin depigmentation results from autoimmune loss of melanocytes from the interfollicular epidermis and hair follicles (Nordlund et al., 2006). Epidemiologic and genetic studies have shown that generalized vitiligo behaves as a multifactorial, polygenic 'complex trait', about 20% of probands reporting one or more affected relatives, typically with a non-Mendelian pattern of inheritance (Alkhateeb et al., 2003; Laberge et al., 2005). Several genes involved in immune function, including loci in the MHC, *CTLA4*, *PTPN22*, *IL10*, *MBL2*, and *NALP1* (*NLRP1*), have been implicated in susceptibility to generalized vitiligo on the basis of genetic linkage or association studies (Spritz, 2007, 2008), particularly in patients and families that also segregate other autoimmune diseases that are epidemiologically associated with vitiligo, principally autoimmune thyroid disease, latent autoimmune diabetes of the adult (LADA), rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease (Alkhateeb et al., 2003; Laberge et al., 2005).

The *CTLA4* gene encodes cytotoxic T lymphocyte antigen 4, a key negative feedback regulator of T-cell activation and proliferation during the immune response (Brand et al., 2005; Gough et al., 2005). Variation in *CTLA4* has been genetically associated with a number of autoimmune diseases, typically at an effect size (odds ratio; OR) of about 1.5 to 2.0 (Brand et al., 2005; Gough et al., 2005; Kavvoura et al., 2007). However, studies of genetic association between *CTLA4* and vitiligo have yielded conflicting results. An initial study of a single *CTLA4* microsatellite in United Kingdom (UK) Caucasian (CEU) patients reported genetic association principally in patients with other autoimmune diseases (Kemp et al., 1999). Similar findings were reported in a very small study of Turkish patients (Itirli et al., 2005). A subsequent study of UK CEU patients observed association of *CTLA4* single-nucleotide polymorphisms (SNPs) only in those vitiligo patients who had other concomitant autoimmune diseases (Blomhoff et al., 2005). In contrast, in a large family-based association study of USA and UK CEU families with generalized vitiligo and other vitiligo-associated autoimmune diseases we found no genetic association with *CTLA4* SNPs (Laberge et al., 2008).

Here, we describe two additional genetic association studies of *CTLA4* SNPs with generalized vitiligo, in two independent Romanian CEU case-control cohorts. Neither individual study showed any evidence of association of *CTLA4* with generalized vitiligo, either in the total patient group or in the subgroup of patients who had other concomitant autoimmune diseases. To resolve the apparent conflicting results among the various genetic association studies of *CTLA4* and vitiligo, we carried out a meta-analysis that combined these two new studies with the two other published association studies of *CTLA4* SNPs with vitiligo in CEU cohorts. This meta-analysis provided much higher statistical power than any of the individual studies, but again showed no genetic association of *CTLA4* SNPs with vitiligo overall. However, in the subgroup of vitiligo patients who had other concomitant autoimmune diseases there was significant association of *CTLA4* SNP rs231775 and near-significance of several other *CTLA4* SNPs in linkage disequilibrium (LD) with rs231775.

Results and discussion

We first carried out two independent case-control association studies of *CTLA4* SNPs, in two independent Romanian case-control cohorts. The first Romanian cohort consisted of 66 unrelated CEU patients with generalized vitiligo (18 with other concomitant autoimmune diseases) and 85 unrelated CEU controls with no auto-immune diseases; details of this case-control cohort have been published previously (Jin et al., 2007). The second Romanian case-control cohort consisted of 101 unrelated CEU patients and 100 unrelated healthy CEU controls from the same geographical region. Among the 101 patients in the second cohort, 90 had vitiligo

vulgaris, 9 acrofacial vitiligo, and 2 vitiligo universalis, and 32 (31.8%) also had other concomitant autoimmune diseases, including autoimmune thyroid disease (17), rheumatoid arthritis (5), adult-onset insulin-dependent diabetes (5), psoriasis (3), systemic lupus erythematosus (1), and alopecia areata (1). There were no significant differences of gender or age distributions among the patients (48.2 ± 18.5) versus controls (47.8 ± 18.7).

In the first case-control cohort, we genotyped seven SNPs spanning 77 kb in the *CTLA4* region of chromosome 2; six SNPs (rs1863800, rs231775, rs3087243, rs11571302, rs11571297, and rs10932037) were genotyped directly and one (rs231806) was imputed based on LD patterns (Marchini et al., 2007). None of these *CTLA4* SNPs deviated from Hardy–Weinberg equilibrium among the controls (not shown). None of these SNPs showed statistically significant (defined as nominal P-value <0.05) allelic (Table 1) or genotypic (not shown) association with generalized vitiligo in either the total patient group ($n = 66$) or in subgroups of patients who had ($n = 18$) or did not have ($n = 48$) other autoimmune diseases. No multiple testing correction was applied, given the lack of any apparent association. Six of the seven SNPs tested were in a single block of LD ($D' > 0.8$), and we observed no significant association of *CTLA4* SNP haplotypes with disease in any patient group ($P = 0.15$; not shown).

In the second case-control cohort we genotyped only SNP rs231775, and we again found no significant allelic or genotypic association with vitiligo overall. Nevertheless, in the subgroup of patients who had other concomitant autoimmune diseases ($n = 32$) allelic association approached statistical significance ($P = 0.06$), at an OR of 1.69 (95% CI: 0.87–2.05).

Blomhoff et al. (2005) reported significant association ($P < 0.03$) of *CTLA4* SNPs in that subgroup of vitiligo patients who had other concomitant autoimmune diseases at ORs > 2.1 . However, initial reports of genetic association tend to over-estimate ORs, resulting in over-estimates of statistical power of subsequent studies (Goring et al., 2001). The two present studies had estimated 80% power to detect significant association ($P < 0.05$) at ORs > 1.8 – 1.9 . To enhance statistical power, we carried out a meta-analysis that combined data from these two present studies with those of the two previously-published studies of *CTLA4* SNPs in CEU cohorts (Blomhoff et al., 2005; Laberge et al., 2008). SNP rs231775 was genotyped in all four studies. SNPs rs231806, rs3087243, rs11571302, and rs11571297 were genotyped by Blomhoff et al. (2005), Laberge et al. (2008), and the first of the two present studies. Two other published studies (Itirli et al., 2005; Kemp et al., 1999) analyzed an un-annotated (AT)_n *CTLA4* microsatellite; each reported association with a different allele, and thus cannot be reconciled with each other and furthermore cannot be statistically combined with SNP-based studies in a meta-analysis.

Considering the four studies of vitiligo in CEU patients that utilized SNPs and which thus can be combined in a meta-analysis, we found no significant heterogeneity across these studies based on Cochran Q-tests of heterogeneity ($P > 0.1$) and based on I^2 tests of inconsistency ($I^2 < 50\%$) applied to each comparison and to each SNP (Table 2). Meta-analysis combining data from these four studies provided greatly enhanced statistical power. For SNP rs231775, the only SNP genotyped in all four studies, the meta-analysis provided 80% power for all comparisons at threshold $P < 0.05$ and OR 1.4, but still showed no association of rs231775 with vitiligo in either the total patient group or the subgroup of patients having only vitiligo, versus controls (Table 2). However, rs231775 showed significant association in the comparison of the subgroup of vitiligo patients who had other concomitant autoimmune diseases, versus controls ($P = 0.01$), and in the comparison of vitiligo patients who had other concomitant diseases, versus patients who had only vitiligo ($P = 0.03$), under the random-effects model. Cumulative meta-analysis of the allelic contrast (G versus A) for rs231775 showed progressive chronological decline in random effect pooled ORs in the comparison of vitiligo patients who had other concomitant autoimmune disease, versus controls, from 1.7 in the earliest study

(Blomhoff et al., 2005) to 1.4 in the meta-analysis that combined all four studies. These results are indicative of the 'winner's curse', the tendency of initial significant genetic association studies to over-estimate the corresponding OR (Goring et al., 2001).

SNPs rs231806, rs3087243, rs11571302, and rs11571297, all of which are in LD ($D' > 0.8$) with SNP rs231775, were tested in three studies. Meta-analysis combining these data provided $>80\%$ power to detect association at ORs ranging from 1.25 comparing all vitiligo patients versus controls, to 1.5 comparing the subgroup of vitiligo patients with other concomitant autoimmune diseases versus controls or versus patients with isolated vitiligo. Nevertheless, meta-analysis showed no significant association of any of these SNPs in any comparison under the more conservative random-effects model (Table 2), but all showed trends toward significance (P -values 0.05–0.10) in the comparison of vitiligo patients with other concomitant autoimmune disease versus controls. Similarly, SNPs rs11571302 and rs11571297 showed trends toward significant association in the comparison of vitiligo patients with other concomitant autoimmune diseases versus those patients having only vitiligo.

These results of the meta-analysis, showing significant association of *CTLA4* SNP rs231775 with vitiligo only in the subgroup of patients with vitiligo plus other concomitant autoimmune diseases, versus controls, and near-significant association of other SNPs in LD with rs231775 in the same patient subgroup, are generally consistent with one of two microsatellite-based studies that could not be combined with the SNP-based studies in a meta-analysis. Kemp et al. (1999) found that the 106-bp allele of a *CTLA4* intragenic (AT)_n microsatellite was marginally associated (uncorrected $P = 0.02$) with vitiligo in the total patient group, but showed much stronger association (uncorrected $P = 0.0001$) in the subgroup of patients with other concomitant autoimmune diseases. The 106-bp allele of this microsatellite is in strong LD with the high-risk G allele of SNP rs231775 (Holopainen and Partanen, 2001), and both of these variants have been genetically associated with a number of other autoimmune diseases.

The high-risk G allele of SNP rs231775 (+49A/G) results in the substitution of threonine by alanine at codon 17 of the leader sequence of the *CTLA4* polypeptide, possibly affecting conformation of the leader peptide resulting in altered intracellular *CTLA4* trafficking. This variant has been correlated with decreased negative regulation of T-cell proliferation, and is thereby thought to predispose to the development of autoimmune diseases (Brand et al., 2005; Gough et al., 2005). Our meta-analysis showed no apparent association of *CTLA4* SNPs with generalized vitiligo overall, but showed significant association of the high-risk G allele of SNP rs231775 in that subgroup of vitiligo patients who had other concomitant autoimmune diseases. The OR for this association was 1.4 (95% CI: 1.05–1.90), comparable to the ORs from meta-analyses for other autoimmune diseases that have primary genetic association with *CTLA4* SNP rs231775 (Brand et al., 2005; Gough et al., 2005). Furthermore, SNP rs231775 showed a significant difference in the comparison of patients with vitiligo and other concomitant autoimmune diseases versus patients who had only vitiligo, indicating that there is a clear difference between these two patient subgroups with respect to *CTLA4*. Overall, these results suggest that *CTLA4* is not genetically associated with vitiligo per se, and that the weak association of *CTLA4* with vitiligo is secondary, reflecting epidemiologic association of vitiligo with other autoimmune diseases that have primary genetic association with *CTLA4*.

Material and methods

Study subjects

The first Romanian CEU vitiligo case-control cohort has been described previously (Jin et al., 2007). Of the second Romanian CEU vitiligo case-control cohort, subjects were recruited at Zalău Hospital between January 2001 and 2002; all were from Salaj, Cluj and Maramures counties in Romania, and all were examined by SAB. Controls had no vitiligo or any other

autoimmune disease. Informed consent was obtained from all subjects. The study was conducted according to the Declaration of Helsinki Principles and was approved by the Ethics Committee of 'Iuliu Hatieganu' University of Medicine and Pharmacy, Cluj-Napoca, Romania and by COMIRB at the University of Colorado Denver.

Samples and genotyping

In the first cohort, we genotyped six SNPs (rs1863800, rs231775, rs3087243, rs11571302, rs11571297, rs10932037) and in the second cohort we genotyped one SNP, rs231775, as described previously (Vaydia et al., 2000; Laberge et al., 2008). In addition, in the first Romanian cohort and in the 126 USA-UK families studied by Laberge et al. (2008), genotypes for SNP rs231806 were imputed using IMPUTE (Marchini et al., 2007), which calculates the probability of missing genotypes using a Bayesian approach based on data from genotyped SNPs, HapMap haplotype data, and estimates of a fine-scale recombination map. All rs231806 imputed genotypes had a maximum posterior genotype probability of >0.95.

Statistical analyses

For each marker tests of allelic association were carried out using Fisher's exact test, and genotypic association was calculated using the Freeman-Halton extension of Fisher's exact test for a 2×3 contingency table (Freeman and Halton, 1951). The significance threshold for nominal P-values was 0.05. Haplotype analysis was performed using Unphased, version 3.0.10, MRC Biostatistics Unit, Cambridge, UK (Dudbridge, 2003). Haplotype frequencies for the phase-unknown Romanian samples were calculated using the expectation maximization algorithm, and haplotype association tests were performed using a chi-square likelihood ratio test. Tests of deviations from Hardy-Weinberg equilibrium among the controls, and calculation of LD between *CTLA4* SNPs, were carried out using Haploview, version 3.32 (Barrett et al., 2005).

Meta-analysis of SNPs rs231806, rs3087243, rs11571302, and rs11571297 incorporated data from two case-control studies (Blom-hoff et al., 2005 and the first current study) and one family-based study (Laberge et al., 2008). Meta-analysis of SNP rs231775 additionally included the second current study. Meta-analysis was performed using STATA 10 (<http://www.stata.com>), testing both a fixed effect model (Mantel-Haenszel method) and random effect model (DerSimonian and Laird method) (Zintzaras, 2006). The ORs, standard errors and corresponding 95% CI were calculated for each study and each SNP, and pooled ORs were then calculated by combining data from case-control and family-based studies as described (Evangelou et al., 2006), weighting individual ORs by the inverse of their variances. P-values < 0.05 were considered statistically significant. We assessed heterogeneity within and between studies using Cochran's Q-statistic (Zintzaras, 2006), weighting the contribution of each study as in the meta-analysis and obtaining P-values for heterogeneity from a chi-square distribution with $k - 1$ degrees of freedom (where k is the number of studies) (Lau et al., 1997). Because of the low sensitivity of the heterogeneity test, the threshold for significance was taken as $P < 0.10$ (Lau et al., 1997).

We measured the degree of inconsistency across studies by calculating the percentage of total between-study variation because of heterogeneity rather than random variation as the I^2 metric using the formula $I^2 = Q - \text{d.f.}/Q$, considering $I^2 = 1-24\%$ as low heterogeneity, $I^2 = 25-49\%$ as moderate heterogeneity, $I^2 = 50-74\%$ as large heterogeneity, and $I^2 \geq 75\%$ as extreme heterogeneity (Evangelou et al., 2006). Cumulative meta-analysis was carried out to evaluate the trend of pooled ORs over time for the contrast between the respective high-risk and low-risk alleles (Zintzaras, 2006).

The power of individual studies and the power of the meta-analysis were computed as the probability of detecting a genetic association with vitiligo at a one-tailed significance level $\alpha = 0.05$, using the formula $P = 1 - \Phi(c_\alpha - \lambda)$, where $\Phi(x)$ is the standard normal cumulative distribution function, c_α is the $100(1 - \alpha)$ percentile of the standard normal distribution and λ is the ratio of $\ln(\text{OR})$ and the sampling variance of $\ln(\text{OR})$ (Hedges and Pigott, 2001).

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Table 1
Allelic association of *CTLA4* SNPs in Romanian vitiligo cases and controls

SNP ^a	controls n (%) ^b	All vitiligo vs. controls			Isolated vitiligo vs. controls			Vitiligo + AI vs. controls			Vitiligo + AI vs. isolated vitiligo		
		n (%) ^b	P	OR (95% CI)	n (%) ^b	P	OR (95% CI)	n (%) ^b	P	OR (95% CI)	P	OR (95% CI)	
Study 1													
rs1863800 [<u>G/A</u>]	104 (61)	87 (67)	0.18	1.29 (0.80–2.07)	65 (69)	0.12	1.42 (0.83–2.43)	22 (61)	0.57	1.00 (0.48–2.08)	0.25	0.70 (0.31–1.56)	
rs231806 [<u>G/C</u>]	94 (56)	81 (62)	0.16	1.30 (0.81–2.07)	60 (64)	0.13	1.39 (0.83–2.33)	21 (58)	0.47	1.10 (0.53–2.28)	0.35	0.79 (0.36–1.74)	
rs231775 [<u>G/A</u>]	63 (37)	56 (44)	0.15	1.32 (0.83– 2.1)	41 (45)	0.15	1.36 (0.81–2.29)	15 (42)	0.37	1.21 (0.60–2.52)	0.46	0.89 (0.4–1.94)	
rs3087243 [<u>G/A</u>]	97 (58)	84 (65)	0.14	1.34 (0.83–2.14)	62 (66)	0.12	1.42 (0.83–2.39)	22 (61)	0.43	1.15 (0.55–2.40)	0.37	0.81 (0.37–1.79)	
rs11571302 [<u>G/T</u>]	98 (58)	81 (63)	0.19	1.27 (0.79–2.03)	59 (64)	0.19	1.31 (0.77–2.21)	22 (61)	0.42	1.15 (0.55–2.40)	0.45	0.87 (0.40–1.94)	
rs11571297 [<u>T/C</u>]	96 (57)	80 (62)	0.22	1.23 (0.77–1.96)	58 (62)	0.24	1.24 (0.74–2.08)	22 (61)	0.37	1.21 (0.58–2.53)	0.55	0.97 (0.44–2.15)	
rs10932037 [<u>T/C</u>]	16 (9)	15 (11)	0.36	1.23 (0.59–2.60)	11 (12)	0.37	1.25 (0.55–2.80)	4 (11)	0.47	1.20 (0.38–3.84)	0.61	0.97 (0.29–3.2)	
Study 2													
rs231775 [<u>G/A</u>]	55 (28)	68 (37)	0.10	1.34 (0.87–2.05)	43 (31)	0.271	1.19 (0.74–1.91)	25 (39)	0.056	1.69 (0.94–3.05)	0.17	1.41 (0.76–2.63)	

^aFor each SNP the high-risk allele is underlined; for *rs231806*, *rs231775*, *rs3087243*, *rs11571302*, and *rs11571297* these were the same as the high-risk alleles assigned by Blomhoff et al. (2005). For *rs1863800* and *rs10932037*, the high-risk allele was assigned as that with the higher allele frequency in patients than in controls. OR odds ratio; CI confidence interval; vs., versus; vitiligo + AI, vitiligo patients with other concomitant autoimmune diseases. P-value based on Fisher's exact test for a 2 × 2 contingency table.

^bHigh-risk allele count (percentage).

Table 2
Pooled ORs for association of *CTLA4* SNP alleles with vitiligo under fixed- and random-effects models

SNP	Study groups			
	All vitiligo vs. controls	Isolated vitiligo vs. controls	Vitiligo + AI vs. controls	Vitiligo + AI vs. isolated vitiligo
rs231806				
cOR	1.12	1.07	1.39	1.29
p ¹	0.1	0.25	0.04	0.06
p ²	0.1	0.25	0.09	0.13
p ³	0.52	0.54	0.14	0.17
I ² (%)	0	0	48.9	43.3
rs 231775				
cOR	1.09	1.04	1.41	1.37
p ¹	0.15	0.34	0.01	0.02
p ²	0.15	0.35	0.01	0.03
p ³	0.47	0.28	0.72	0.31
I ² (%)	0	22.4	0	16.1
rs3087243				
cOR	1.07	1.02	1.32	1.32
p ¹	0.18	0.44	0.05	0.05
p ²	0.18	0.42	0.1	0.13
p ³	0.55	0.26	0.28	0.14
I ² (%)	0	24.2	21.38	47.36
rs11571302				
cOR	1.06	1.01	1.35	1.35
p ¹	0.23	0.42	0.04	0.04
p ²	0.23	0.42	0.06	0.1
p ³	0.65	0.52	0.25	0.17
I ² (%)	0	0	26.6	41.8
rs11571297				
cOR	1.04	0.99	1.28	1.29
p ¹	0.31	0.47	0.06	0.05
p ²	0.31	0.47	0.06	0.05
p ³	0.75	0.5	0.64	0.44
I ² (%)	0	0	0	0

Vitiligo + AI vitiligo with other concomitant autoimmune diseases; cOR combined OR from random-effects model; vs versus; p¹ Mantel-Haentzel method testing a fixed-effects model; p² DerSimonian and Laird method testing a random-effects model; p³ Cochran Q-test of heterogeneity; I² Test of inconsistency; when negative was set to 0.