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Lack of association of functional *CTLA4* polymorphisms with juvenile idiopathic arthritis

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Abstract

Objectives—Juvenile idiopathic arthritis (JIA) is an autoimmune disorder mediated by Th1-immune responses. Cytotoxic T-lymphocyte Antigen 4 (CTLA4), expressed on the T-cell surface, plays a negative role in regulating T-cell activation. Single nucleotide polymorphisms (SNPs) in *CTLA4* have been implicated in susceptibility to several autoimmune disorders, including JIA. Our objective was to test three functional *CTLA4* variants for association with JIA.

Methods—Families of 531 children with JIA were genotyped for SNPs located in the promoter region (C-318T), exon-1 (A49G), and the 3' untranslated region (CT60) of *CTLA4* by PCR amplification and digestion. Family-based association test (FBAT) was used to test *CTLA4* SNPs and haplotypes for association with JIA. A second independent cohort of more than 300 children with JIA and 500 controls were genotyped for case-control analyses. Case-control analyses of the combined cohorts, as well as meta-analyses of published association studies between *CTLA4* and JIA, were performed.

Results—There were no deviations of transmission of any of the CTLA4 variants to children with JIA, or JIA subtypes, by FBAT. There were also no significant associations between *CTLA4 C-318T* or *A49G* SNPs in 650 JIA cases and 350 controls. Similarly, there were also no significant associations between *CT60* variants with over 800 JIA cases and 500 controls. The meta-analysis also failed to confirm an association between JIA and *CTLA4* variants.

Conclusions—These results suggest that *C-318T*, *A49G* or *CT60* or haplotypes tagged by these CTLA4 SNPs are not associated with JIA or major JIA subtypes.

Keywords

CTLA4; JRA; genetics; autoimmune; association; juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) comprises a group of chronic childhood arthritides [1]. Although associations between JIA and variants in the major histocompatibility complex (MHC) have been described, non-MHC variants, such as *PTPN22* also play a role in JIA susceptibility. The pathogenesis of JIA is mediated, in part, by T-cells [2]. The synovial tissue

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contains a high proportion of activated T-cells. Hence genes encoding proteins that regulate the interactions between T-cells and antigen-presenting cells are good candidates for genetic association studies of JIA.

CTLA4 (CD152) is a T-cell surface molecule that negatively regulates T-cell activation[3]. Three single nucleotide polymorphisms (SNPs) of *CTLA4* (C-318T rs5742909, A49G rs231775, and CT60 rs3087243) are associated with many autoimmune disorders, including type-1 diabetes (T1D), rheumatoid arthritis (RA) and autoimmune thyroiditis. To date three association studies investigating *CTLA4* variants and JIA have been published with mixed results [4-6]. Our objectives were to determine if these functional *CTLA4* variants are associated with JIA susceptibility, and to replicate an association reported earlier between C-318T and juvenile rheumatoid arthritis (JRA) [4].

Patients and methods

Cohort 1 consisted of 531 children with JIA (143 persistent oligoarticular, 69 extended oligoarticular, 165 rheumatoid factor (RF)-negative polyarticular, 44 RF-positive polyarticular, 91 systemic, and 19 other JIA subtypes) and their families in part from a registry sponsored by the National Institute of Arthritis and Musculoskeletal and Skin Diseases and the Cincinnati Children's Hospital Medical Center. The median onset age was 5.5 years, and 73% of the subjects were female. Cohort 2 comprised 365 children with JIA (134 persistent oligoarticular, 27 extended oligoarticular, 82 RF-negative polyarticular, 32 RF-positive polyarticular, 32 systemic, 27 enthesitis related arthritis and 31 other JIA subtypes) from the Intermountain States Database of Childhood Rheumatic Diseases. The median onset age was 5.6 years, and 66% of the subjects were female. Additionally, 551 adult controls screened for several common autoimmune diseases were ascertained from Salt Lake City, UT. All cases and controls from cohort 2 were used for CT60 genotyping, since this SNP shows the strongest association with autoimmunity [7]. Cases, family members and controls were predominantly (>90%) of Northern European ancestry. Subjects were enrolled under protocols approved by the Institutional Review Boards at the Cincinnati Children's Hospital Medical Center and the University of Utah.

Genotyping

Subjects were genotyped for C-318T and A49G, as follows. Polymerase chain reaction (PCR) amplification of genomic DNA was performed using these primers: AGGGCTCAGAAAGTTAGCAGCCT and AATACAGAGCCAGCCAAGCCAGAT. Cycling conditions were: 94°C for 15 minutes, followed by 40 cycles: 94°C for 30 seconds, annealing at 62°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. After PCR amplification, the product was digested separately with Mse1 and Tse1. CT60 was genotyped as previously described, using the primers AGTGCTTGATTGCGTGG and TGCTGAGACTATACATTGGTTAAG, and HpyCH4IV for digestion [8].

Analysis

The program Haploview was used to test for Hardy-Weinberg Equilibrium (HWE) and to infer haplotypes [9]. SNPs, haplotypes and genotypes were tested for association with JIA in cohort 1 by a transmission-disequilibrium test, implemented in the family-based association test (FBAT) program [10]. Briefly, FBAT calculates a transmission score for each informative family. FBAT determines the expected scores under the null hypothesis of no association or linkage by conditioning on offspring trait values and parental genotypes, assuming Mendelian transmission of alleles to offspring. The difference between the observed and expected score is summed over all families to generate a test statistic, which has a chi-square distribution.

FBAT allows for incorporation of different genetic models. Both SNPs and haplotypes can be analyzed.

Case-control analyses were performed to investigate if alleles, genotypes or haplotypes of CTLA4 were associated with JIA. Haplotype logistic regression was also performed to test CTLA4 haplotypes for association with JIA. Published association studies of CTLA4 and JIA were identified using a PUBMED search[4-6], and allele frequency data was obtained from the individual studies. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for each SNP. The pooled OR was calculated under a fixed effects model, weighting the studies by sample-size. Mantel-Haenszel Chi-square tests were performed for the individual studies and the studies combined. The Breslow-Day test was performed for heterogeneity among the studies, with significance set at $p < 0.05$. Analyses were performed using SAS 9.1.

RESULTS

Allele and haplotype frequencies of the three polymorphisms

The minor allele frequencies (MAF) of the C-318T, A49G and CT60 SNP among the unaffected parents were 0.09, 0.39 and 0.43 respectively. MAF of these three SNPs in controls were 0.1, 0.39 and 0.44 respectively. These frequencies are similar to the observed MAF of 0.09, 0.36 and 0.48 respectively in 652 “White” controls in the study by Ueda et al[7]. None of the SNPs deviated from HWE. Three-locus haplotype frequencies in parents were as follows: CAA = 0.41, CGG = 0.38, CAG = 0.11, TAG = 0.08 and CGA = 0.02. The three SNPs were in high linkage disequilibrium with each other (D’ 0.8 to 1.0). Controls had similar haplotype frequencies.

Tests of association between CTLA4 SNPs and JIA

No deviation of transmission of any of the CTLA4 SNPs to JIA probands was observed under an additive model using FBAT (Table 1). Analyses under other models, (dominant, recessive, or genotypic) also did not reveal any associations in JIA probands. Analyses performed after stratifying by JIA subtypes revealed an association between C-318T and RF-positive polyarticular JIA ($p < 0.04$). For the RF-positive poly JIA families, under a genotypic model, there was a borderline association with the A49G-G/G genotype (12 informative families; observed score (S) = 8, expected score (E(S)) = 5; $p < 0.07$) and for the CT60-G/G genotype (12 families, S = 8, E(S) = 4.8; $p < 0.05$). Other JIA subtypes revealed no associations. There were no transmission deviations of CTLA4 haplotypes to probands with JIA or any JIA subtype, including the haplotype (G-G) containing the susceptibility alleles at both A49G and CT60.

Case-control analysis using cohort 2 revealed no associations between CTLA4 variants and JIA. Case-control analyses were repeated after combining the JIA cases from both cohorts. No associations were found between C-318T or A49G variants and JIA or JIA subtypes, using 650 cases and 345 controls (Table 2). Similarly the CT60 variants showed no associations in 818 cases and 518 controls. Logistic regression analyses did not demonstrate an association between CTLA4 haplotypes and JIA.

Meta-analyses of C-318T and A49G SNPs and JIA

Cases and controls were mostly of European descent, from Germany [4,5], Northern Ireland [6] and the United States (present study). Allele frequency information was available on 1915 individuals (933 cases, 982 controls) for the analysis of C-318T (Table 3). The pooled OR was not significant (1.01, 95% CI 0.80-1.27). There was significant heterogeneity between the studies ($p <0.01$), mainly due to the study by Miterski et al, which found an association in the opposite direction compared to the remaining studies [4]. Excluding that study resulted in a

pooled OR of 0.73 (0.55-0.97, p <0.03) for the presence of the T allele. Allele frequency information was available on 2457 individuals (1005 cases, 1452 controls) for the A49G analysis. There was no heterogeneity between studies (p >0.20). The pooled OR was not significant 0.96(0.84-1.09). The allele frequencies of the CT60 SNP were not published by Suppiah, et al. [6], precluding a meta-analysis of SNP CT60.

DISCUSSION

CTLA4, expressed on activated T-cells negatively regulates T-cell activation [3]. Functional *CTLA4* variants could contribute to the pathogenesis of disorders characterized by abnormal T-cell responses, including JIA. *CTLA4* variants are associated with T1D, autoimmune thyroiditis, and RA [7,11-13]. Variants tested in our study have functional consequences. For instance, the T allele at *C-318T* is associated with up-regulation of *CTLA4* transcription [14]. The *A49G* SNP, which changes threonine to alanine at position 17 in the leader sequence of CTLA4, affects the CTLA4-driven down-regulation of T-cell activation [15]. The strongest signal of association in the *CTLA4* locus is with the CT60 SNP [7]. In a comprehensive study of CTLA4 variants, Ueda et al. demonstrated that the G allele at CT60 was associated with susceptibility to autoimmunity. The ratio of soluble CTLA4 to full length CTLA4 in CD4 T-cells was 50% lower in individuals with the susceptible genotype (G/G) compared with individuals who had the A/A genotype ($p < 0.002$). Thus, CTLA4 variants investigated in our study have functional consequences and known disease associations.

To date three studies have investigated associations between *CTLA4* variants and juvenile arthritis[4-6]. These studies were primarily focused on adult RA or asthma patients and included relatively small numbers of children with JIA. Only limited phenotypic information about JIA/JRA was available in these studies. Miterski et al. reported an association of C318T with JRA in a study of 200 subjects from Germany [4]. An association with A49G was not observed. In another study, Suppiah et al. tested 72 children with JIA, 289 adults with RA, and 475 controls from Northern Ireland for CTLA4 variants[6]. In contrast to other studies on RA, Suppiah et al found an increased frequency of the A allele of the A49G SNP in the RA group and in the combined arthritis group, although an association was not found with the JIA subgroup alone. That study did not find any associations with the CT60 SNP, although they found differences in frequencies of A49G-CT60 haplotypes between arthritic cases and controls. Finally, Schubert et al. studied C-318T and A49G SNPs in a cohort of 321 children with asthma, 86 children with JIA, and 270 controls from Germany[5]. That study found no associations between JIA and either the C-318T or A49G variants.

We did not find an association between C–318T, A49G, or CT60 variants and JIA in a large cohort. We also did not replicate the association described between C-318T and JRA by Miterski et al. Lack of replication of genetic studies occurs due to myriad reasons, including type-1 statistical errors or hidden population stratification in the original study, inadequate power in replication studies, and population differences. The discrepancies in the reported results for C-318T in JIA could be due to true population differences or co-occurrence of other autoimmune disorders in the cohort studied by Miterski et al [4]. The mean age of disease onset (10.3 years) was higher in the cohort studied by Miterski et al. than in our study, raising the possibility that there were more individuals with RF-positive JIA in their cohort.

Inadequate power can cause failure to replicate, but our combined cohort is among the largest used for JIA genetic studies. Our study had 80% power to detect OR of 1.8, 1.5 and 1.4 for C-318T, A49G and CT60 respectively. Furthermore, our meta-analysis failed to find an association between either variant and JIA. The meta-analysis had 80% power to detect an OR of 1.53 and 1.27 for C-318T and A49G respectively, but an association of lower magnitude cannot be ruled out, especially for C-318T. We did observe an uncorrected $P < 0.05$ for C-318T

when we combined our cohort and the cohort reported by Schubert et al. [5], but this result suggests that the T allele is protective, in contrast to the finding by Miterski et al that it increases susceptibility. Although this finding might reflect a statistical fluctuation, it would be interesting if this observation can be replicated in other JIA cohorts because the T allele at C-318T has been suggested to protect against autoimmune diseases by increasing CTLA4 transcriptional activity [14].

CTLA4 variants are associated with adult RA. However, a meta-analysis found that the association between the *CT60-G* allele and RA was relatively modest, with an OR of ~1.1 [13]. When RA patients were stratified, only patients positive for anti-citrullinated cyclic peptide (CCP) antibodies demonstrated a significant association with the CT60 variant, while CCP-negative patients did not show an association [13]. Only a small proportion of children with JIA have RF or anti-CCP antibodies, and this could explain the lack of association of *CTLA4* variants with JIA. Although our RF-positive polyarticular JIA cohort was small, borderline associations were observed between this subtype and both the C-318T and A49G polymorphisms. Furthermore, FBAT revealed an association between the CT60-G/G genotype and RF-positive polyarticular JIA. These observations suggest that while RF-positive polyarticular JIA might share genetic associations with adult RA, the other JIA subtypes are likely distinct phenotypes and consequently have different genetic associations. It is conceivable that genetic factors associated with RF-negative RA are associated with JIA.

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Table 1

Family based association tests under an additive genetic model for C-318T, A49G and CT60 variants in JIA families.

Variant[1]	Phenotype	N[2]	Allele 1		Allele 2		Var (S)[5]	Z[6]	P
			S[3]	E(S)[4]	S[3]	E(S)[4]			
C-318T	All JIA	107	157	156.2	57	57.8	29.5	0.15	0.88
C-318T	Persistent Oligo	33	50	47.5	16	18.5	9.8	0.80	0.42
C-318T	Extended Oligo	13	20	19.5	6	6.5	3.3	0.28	0.78
C-318T	Polyarticular	29	44	42.5	14	15.5	8.8	0.50	0.61
C-318T	Polyarticular RF+	8	9	11.8	7	4.2	1.9	2.10	0.04
C-318T	Systemic	18	25	25.8	11	10.2	4.4	0.40	0.69
C-318T	Other	5	8	7.5	2	2.5	1.25	0.45	0.65
A49G	All JIA	228	274	267.3	182	188.7	92.0	0.69	0.49
A49G	Persistent Oligo	63	72	70.8	54	55.2	26.9	0.22	0.82
A49G	Extended Oligo	31	39	36	23	26	11.5	0.89	0.38
A49G	Polyarticular	65	81	82.5	49	47.5	23.5	0.31	0.76
A49G	Polyarticular RF+	22	22	23.75	22	20.25	8.3	0.61	0.54
A49G	Systemic	39	51	46.75	27	31.25	17.6	1.01	0.31
A49G	Other	8	9	8	7	8	3.5	0.54	0.59
CT60	All JIA	181	157	163.1	205	198.9	67.2	0.75	0.46
CT60	Persistent Oligo	55	50	49.4	60	60.6	20.3	0.14	0.89
CT60	Extended Oligo	28	24	25.25	32	30.75	10.7	0.38	0.70
CT60	Polyarticular	48	41	46.6	55	49.4	18.2	1.30	0.19
CT60	Polyarticular RF+	13	7	10.8	19	15.2	5.7	1.60	0.11
CT60	Systemic	29	27	24.1	31	33.9	10.3	0.91	0.36
CT60	Other	7	7	6.5	7	7.5	1.75	0.38	0.71
C-G-G	All JIA	160	187.9	185.2	-	-	65.4	0.33	0.74

[1] For the C-318T SNP, allele 1 was -318C and allele 2 was -318T. For the A49G SNP, allele 1 was 49A, and allele 2 was 49G. For the CT60 SNP, allele 1 was CT60-A and allele 2 was CT60-G.

[2] N: Number of informative families (i.e., families with at least one heterozygous patient).

[3] S: Test statistic from family-based association test for the observed number transmitted alleles.

[4] E(S): Expected value of S under the null hypothesis (no linkage or association)

[5] Var(S): Empirical variance

[6] Z: Z score for each allele.

Table 2

Results of case-control analysis of C-318T, A49G and CT60 variants and JIA

Variant	Phenotype	N	Genotype[1]			Allele[1]		P[2]
			1/1	1/2	2/2	1	2	
C-318T	Controls	350	80.9	17.7	1.4	89.7	10.3	
C-318T	All JIA	650	83.8	15.4	0.8	91.5	8.5	0.20
C-318T	Persistent Oligoarticular	202	81.2	17.8	1.0	90.1	9.9	0.81
C-318T	Extended Oligoarticular	76	86.8	13.2	0.0	93.4	6.6	0.70
C-318T	Polyarticular	197	83.8	15.2	1.0	91.4	8.6	0.43
C-318T	Polyarticular RF+	50	86.0	14.0	0.0	93.0	7.0	0.39
C-318T	Systemic	90	84.4	14.4	1.1	91.7	8.3	0.52
C-318T	Other	35	88.6	11.4	0.0	94.3	5.7	0.31
A49G	Controls	345	36.2	49.6	14.2	61.0	39.0	
A49G	All JIA	650	40.2	44.0	15.8	62.2	37.8	0.65
A49G	Persistent Oligoarticular	206	39.3	42.2	18.4	60.4	39.6	0.81
A49G	Extended Oligoarticular	78	34.6	48.7	16.7	59.0	41.0	0.70
A49G	Polyarticular	194	43.3	45.9	10.8	66.2	33.8	0.10
A49G	Polyarticular RF+	50	28.0	46.0	26.0	51.0	49.0	0.07
A49G	Systemic	88	45.5	36.4	18.2	63.6	36.4	0.58
A49G	Other	34	44.1	50.0	5.9	69.1	30.9	0.23
CT60	Controls	518	18.7	49.6	31.7	43.5	56.5	
CT60	All JIA	818	19.3	46.8	33.9	42.7	57.3	0.68
CT60	Persistent Oligoarticular	257	14.4	50.2	35.4	39.5	60.5	0.13
CT60	Extended Oligoarticular	91	20.9	46.1	33.0	44.0	56.0	0.92
CT60	Polyarticular	221	21.3	44.8	33.9	43.7	56.3	0.96
CT60	Polyarticular RF+	68	11.8	50.0	38.2	36.8	63.2	0.13
CT60	Systemic	107	27.1	43.0	29.9	48.6	51.4	0.17
CT60	Enthesitis related	27	33.3	37.0	24.6	51.9	48.1	0.23
CT60	Other	47	19.2	48.9	31.9	43.6	56.4	0.98

[1] For the C-318T SNP, allele 1 was -318C and allele 2 was -318T. For the A49G SNP, allele 1 was 49A, and allele 2 was 49G. For the CT60 SNP, allele 1 was A and allele 2 was G.

Allele and genotype percents are shown.

[2] P; Significance values for carriage of allele 2 are shown.

Table 3

Results of meta-analysis of CTLA4 C-318T and A49G polymorphisms and JIA

VARIANT	AUTHOR	CASES				CONTROLS				OR[1]	95%CI
		N	1[2]	2[2]	MAF[3]	N	1	2	MAF		
C-318T											
	Miterski	197	342	52	0.13	362	674	50	0.07	2.05	1.36-3.09
	Schubert	86	162	10	0.06	270	480	60	0.12	0.49	0.25-0.99
	Present study	650	1190	110	0.08	350	628	72	0.10	0.80	0.59-1.10
	Pooled[4]	**933**	**1694**	**172**	**0.09**	**982**	**1782**	**182**	**0.09**	**1.01**	**0.80-1.27**
A49G											
	Miterski	197	255	139	0.35	362	455	269	0.37	0.92	0.71-1.2
	Schubert	86	101	71	0.41	270	351	189	0.35	1.30	0.92-1.86
	Suppiah	72	89	55	0.38	475	525	425	0.45	0.76	0.53-1.09
	Present study	650	809	491	0.38	345	421	269	0.39	0.95	0.79-1.15
	Pooled	**1005**	**1254**	**756**	**0.38**	**1452**	**1752**	**1152**	**0.40**	**0.96**	**0.84-1.09**

[1]: OR for carriage of allele 2 are shown.

[2]: For the C-318T SNP, allele 1 was -318C and allele 2 was -318T. For the A49G SNP, allele 1 was 49A, and allele 2 was 49G.

[3]: MAF: Minor allele frequency.

[4]: For the C-318T meta-analysis there was a significant heterogeneity, attributed to the study by Miterski et al. Excluding that study and repeating the meta analysis yielded a pooled OR of 0.73 (0.55 to 0.97, p <0.03).