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SNPs in the *FOXP3* gene region show no association with Juvenile Idiopathic Arthritis in a UK Caucasian population

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Abstract

Objective—A region on the short arm of the X-chromosome, Xp11, has previously been linked to childhood-onset polyarthritis. Mapping to the linked region is *FOXP3*, a transcription factor that regulates regulatory T cell (T_{reg}) development and function. The objective of this study was to determine whether single nucleotide polymorphisms (SNPs) in the *FOXP3* gene region contribute to JIA susceptibility.

Method—Nine *FOXP3* SNPs were genotyped in 761 JIA cases and 402 controls using the Sequenom® MassARRAY® system. Association was measured using either χ^2 or Fisher's exact test at the allelic and genotypic level. Furthermore, cases and controls were stratified by gender and association measured for each stratum.

Results—None of the SNPs showed an association with JIA. Similarly, the lack of association was also evident in both the female and male cohorts.

Conclusion—Although *FOXP3* presents itself as a good candidate for contributing to JIA susceptibility, this study, which was powered to detect associations with genotypic relative risk >2 in the female cohort, has failed to find an association between SNPs in the *FOXP3* gene region and JIA.

Keywords

FOXP3; Juvenile idiopathic arthritis; Association analysis; X-linked gene

Introduction

Juvenile idiopathic arthritis (JIA) refers to a group of diseases, all of which are characterized by chronic inflammation of one or more joints, affecting children of 16 years and under. The cause of JIA is believed to result from the combined action of genetic and environmental factors. The genetic component of JIA has been inferred from twin and affected sibpair studies, with the sibling recurrence risk (λ_s) estimated to be 15 [1]. Like other autoimmune diseases, specific human leukocyte antigen (HLA) alleles have been consistently linked and associated to JIA in a subtype-specific manner [1]. However, HLA-DR accounts for only a small proportion of the JIA susceptibility (~17%) [2], suggesting that additional non-HLA polymorphisms also influence susceptibility [3]. Using a cohort of North American JRA affected sibling pairs, a genome-wide linkage scan identified several peaks of linkage separate from the HLA region. Nominal linkage was identified on the short arm of the X-

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chromosome, Xp11, in an early onset (before sixth birthday) polyarticular course cohort [4]. Interestingly, this peak of nominal linkage overlaps with an X-chromosome autoimmune disease-linked region identified in humans [5]. The evidence for an X-linked susceptibility allele provides tantalising clues as to why females are predominantly affected in several common autoimmune diseases. Indeed, JIA epidemiological evidence shows an increased female prevalence in oligoarticular and polyarticular onset arthritis (2–3:1), but an equal ratio for systemic onset disease [6].

Mapping in the same region as the JIA and autoimmune Xp11 loci is the *FOXP3* gene at Xp11.23. The *FOXP3* gene and its mouse orthologue *scurfin/foxp3* are members of a gene family that encode transcription factors with a forkhead box ('fox') DNA-binding domain. This transcription factor is known to play a crucial role in T-cell regulation and immune homoeostasis. Mutations in the open-reading frame of *FOXP3* are associated with a rare fatal paediatric disorder, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [7], which exhibits aggressive autoimmune features. The transcription factor product regulates the development and function of a subset of CD4+ T-cells expressing CD25 (IL-2 receptor α-chain), known as regulatory T-cells (T_{reg}). T_{reg} suppress the proliferation of autoreactive lymphocytes both in the thymus and the periphery, in cell-cell contact manner [8-10]. Therefore, through mediating T_{reg} development, *FOXP3* contributes towards the natural tolerance to self-antigens.

Investigations into the role of T_{reg} on influencing the immune response in JIA have shown the suppressive T_{reg} to be present in high quantities at sites of local inflammation [11-13]. Persistent oligoarticular, extended oligoarticular and polyarticular JIA patients have an increased concentration of activated T_{reg} in synovial fluid (SF) from inflamed knees compared to peripheral blood (PB) samples [11-13]. *FOXP3* expression is increased in SF-derived T_{reg}, which correlates with increased suppressive activity on effector T-cell proliferation and cytokine production [13].

A single study has implicated a genetic variant within *FOXP3* as contributing to a common autoimmune disease. Bassuny and colleagues [14] reported an association between a microsatellite marker within Intron 0 of *FOXP3* and Type I diabetes in a Japanese population. The associated microsatellite allele significantly increased luciferase expression in several T-cell phenotypes.

Given the evidence above, we hypothesized that common SNPs in the *FOXP3* gene region influence the risk of developing JIA. A population-based case-control association analysis was used to assess the risk of JIA conferred by SNPs in the *FOXP3* gene region.

Materials and methods

Cases and controls

All samples were of UK Caucasian ethnic origin and were recruited with ethical approval and had given their informed consent.

DNA samples were available for 761 JIA cases from the British Society for Paediatric & Adolescent Rheumatology (BSPAR) JIA National Repository held at the Arthritis Research Campaign Epidemiology Unit at The University of Manchester. The repository contains DNA samples from JIA patients recruited from 17 centres across the UK that have all been classified by the International League of Associations for Rheumatology (ILAR) diagnostic criteria [15]. Of the JIA cases, 507 (67%) were girls and the entire cohort had a median onset age of 5 years.

DNA samples from 402 healthy volunteers were available to serve as controls. This cohort was composed of 315 samples from blood donations of healthy adult volunteers from the Oxford, UK region and 87 samples from healthy individuals who have been recruited by General Practitioners to serve as controls for the Norfolk Arthritis Register (NOAR) research [16]. 52% of the controls were females.

SNP selection

The International Haplotype Mapping (HapMap) (www.hapmap.org) and Ensembl (http://www.ensembl.org/index.html) SNP databases were used to select SNPs in the *FOXP3* gene region. The screened region was extended 10 kilobases up-stream of the annotated transcription start site and down-stream at the end of the last *FOXP3* exon. The selection criteria was that SNPs had a minor allele frequency (MAF) >5% in the Utah population of European descent (CEU) and had been validated. Nine SNPs were identified from HapMap (Phase I data, release 16c.1). Using the Ensembl (version 37, February 2006) Biomart Data Mining function and the same SNP criteria, 2 additional SNPs were identified. Therefore, 11 SNPs constituted the selection set. However, nine SNPs were incorporated into the multiplex assay design and taken forward to genotyping.

Genotyping

SNPs were genotyped using the Sequenom® MassARRAY technology (Sequenom®, San Diego, CA, USA, www.sequenom.com). The iPLEX™ assay was followed according to manufacturers instructions (www.sequenom.com) using 5 ng of genomic DNA.

Quality control

The sample quality control (QC) threshold was set at 50% (five out of nine successful genotype calls). The SNP QC threshold was set at 80%. All SNPs were tested for Hardy–Weinberg equilibrium at the 5% significance level in the female cohort. SNPs not in HWE in the female control cohort were excluded from all analyses.

Statistical analysis

All association analyses were carried out by calculating the odds ratio for each SNP marker and significance was determined at the 5% level using either the χ^2 test or Fisher's exact test, as implemented in Stata version 9.0. All *P*-values are two-sided. In the entire JIA cohort (JIA whole), allele-wise association was measured. Gender was taken into account with the males contributing a single allele to the analysis and females contributing two alleles. The JIA cohort was then stratified by gender and tested against corresponding gender specific control groups. For the female cohort, association between genotypes and alleles of SNPs and JIA susceptibility were measured. In the females the influence on disease risk by carriage of either allele was also calculated. For the male analysis only allele-wise association was measured.

Using the female cases and controls only, we had >80% power to detect a genotypic relative risk (GRR) of 2.1 under a dominant inheritance model at the 5% significance level.

Results

Two SNPs, rs2294021 (exact $P = 0.0206$) and rs5906761 (exact $P = 0.0181$), were not in HWE in the female controls and SNP rs3761549 failed the SNP QC (65%). These SNPs were removed from further analysis. In the female control cohort, the remaining six SNPs were in HWE, passed quality control checks and had allele frequencies similar to those published on HapMap and Ensembl.

The impact of common *FOXP3* SNPs on JIA susceptibility was first measured in the JIA whole cohort. None of the SNPs showed evidence of association (Table 1). Likewise, stratifying the JIA cohort and controls by gender failed to detect any evidence of association between the genotyped SNP markers and JIA (Table 2).

Discussion

Our study aimed to evaluate the contribution of single nucleotide polymorphisms in the *FOXP3* gene region towards JIA susceptibility in a population-based case-control association analysis. Our data shows no associations between SNPs in the *FOXP3* gene region and JIA.

FOXP3 is a transcription factor that controls the development of regulatory T cells (T_{reg}), primarily of the CD4+CD25+phenotype [8, 10]. T_{reg} suppress the proliferation and cytokine production of effector T cells by a cell–cell contact dependent, cytokine independent mechanism. Recent analyses of peripheral blood and synovial fluid samples from JIA patients show an increase in the proportion of T_{reg} populating sites of local inflammation with enhanced suppressive capabilities [11-13]. Interestingly, de Kleer *et al.* [11] showed increased *FOXP3* expression is greater in T_{reg} from persistent oligoarticular JIA children than from extended oligoarticular JIA children who exhibit a more severe, less favourable disease course However, we found no significant differences between the subtypes on comparison of genotypes (data not shown).

The *FOXP3* gene is localized in the small arm of the X-chromosome in the vicinity of the region that showed linkage to childhood onset polyarthritis [4, 17, 18]. Accordingly, we tested whether the *FOXP3* SNPs conferred a disease risk restricted to early-onset (before 6 years) polyarticular arthritis (Rheumatoid Factor positive or negative), but found no evidence of association (data not shown). This region has also been linked to other common autoimmune disorders, including type 1 diabetes and rheumatoid arthritis, whilst being identified as an autoimmune locus through meta-analysis [5, 18]. The first indication that *FOXP3* might be responsible for the observed linkage and influence susceptibility to common autoimmune disorders was provided by Bassuny and colleagues, who identified a weak association between a GT15 microsatellite in intron 0 and type 1 diabetes using 199 affected Japanese children and 289 healthy children [14]. However, attempts to confirm this association in independent cohorts of different ethnic origin and other autoimmune diseases have been unsuccessful. Using 418 type 1 diabetic families and a further 268 male patients and 326 healthy males for a case-control analysis from Sardinia, Zavatarri *et al* detected no association with the intron 0 microsatellite, nor with seven SNPs and 4 other microsatellites in the *FOXP3* gene region [19]. Similarly, Sanchez *et al.* [20], were unable to detect an association with the GT microsatellite and several autoimmune diseases although intriguing allele frequency differences were seen between the Spanish and Japanese control subjects. Although such studies focused on microsatellites and are not directly comparable, there exists no evidence of *FOXP3* increasing the risk of developing a common autoimmune disorder within Caucasian populations, which agrees with the findings of this study.

It is important to note that the lack of association in this study does not completely rule out *FOXP3* as a JIA candidate gene. Our study had power to detect a GRR of 2.1 in the female cohort only and obtained data for 81% of the common HapMap SNPs in the *FOXP3* gene region (release 21; phase II July 2006). However, it could be that SNPs in the *FOXP3* gene region confer a smaller risk of developing JIA. Future efforts to identify *FOXP3* SNPs carrying a smaller JIA risk will require a larger sample size than has been used here.

In conclusion, there is currently no genetic evidence to suggest that polymorphisms in the *FOXP3* gene region confer susceptibility to JIA.

Rheumatology key message

- SNPs with a MAF>0.05 in the *FOXP3* gene region show no association with susceptibility to JIA in a UK Caucasian population.

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TABLE 1

JIA whole association analysis

	Controls			Cases			
		Control Allele Frq			Case Allele Frq		
Locus	***N***	**1**	**2**	***N***	**1**	**2**	***P*-value**
rs6609857	364	0.76	0.24	479	0.73	0.27	0.22
rs2294020	357	0.76	0.24	463	0.73	0.27	0.29
rs2280883	363	0.54	0.46	471	0.55	0.45	0.81
rs2232367	371	0.97	0.03	481	0.97	0.03	0.89
rs3761547	353	0.85	0.15	447	0.86	0.14	0.80
rs4824747	362	0.85	0.15	436	0.86	0.14	0.81

N, number of samples in each cohort.

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TABLE 2

Male cohort and Female cohort association analysis

	Males							Females								
	Controls			Cases				Controls				Cases				
		Allele Frq			Allele Frq				Genotype Frq				Genotype Frq			
Locus	***N***	**1**	**2**	***N***	**1**	**2**	***P*-value**	***N***	**11**	**12**	**22**	***N***	**11**	**12**	**22**	**Genotype exact *P***
rs6609857	189	0.76	0.24	154	0.74	0.26	0.64	175	101 (58)	63 (36)	11 (6)	325	166 (51)	140 (43)	19 (6)	0.29
rs2294020	184	0.76	0.24	151	0.77	0.23	0.78	173	100 (58)	62 (36)	11 (6)	312	157 (50)	136 (44)	19 (6)	0.24
rs2280883	186	0.53	0.47	149	0.53	0.47	0.95	177	57 (32)	82 (46)	38 (21)	322	92 (29)	174 (54)	56 (17)	0.24
rs2232367	195	0.97	0.03	156	0.97	0.03	1.00	176	164 (93)	12 (07)	0 (0)	325	305 (94)	20 (6)	0 (0)	0.85
rs3761547	184	0.85	0.15	143	0.87	0.13	0.72	169	122 (72)	44 (26)	3 (2)	304	225 (74)	70 (23)	9 (3)	0.61
rs4824747	186	0.85	0.15	135	0.85	0.15	0.94	176	126 (72)	47 (27)	3 (2)	301	222 (74)	72 (24)	7 (2)	0.78

N, number of samples in each cohort. Percentages are in parentheses

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