Brief Genetics Report

No Association Between Variation of the FOXP3 Gene and Common Type 1 Diabetes in the Sardinian Population

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Mutations of the forkhead/winged helix transcription factor FOXP3 gene on chromosome Xp11.23 cause a rare recessive monogenic disorder called IPEX (immune dysregulation, polyendocrinopathy, including type 1 diabetes, enteropathy, and X-linked syndrome). FOXP3 is necessary for the differentiation of a key immune suppressive subset of T-cells, the CD4+CD25+ regulatory T-cells. Previously, we reported a significant male-female bias in the common, multifactorial form of type 1 diabetes in Sardinia and evidence of linkage of chromosome Xp11 to the disease. These findings indicate that *FOXP3* is a prime functional and positional candidate locus for the common form of type 1 diabetes. In the present study, we initially scanned 82 kb of the *FOXP3* region for common polymorphisms, including sequencing all of the coding and functionally relevant portions of the gene in 64 Sardinian individuals. Then the most informative polymorphisms in 418 type 1 diabetic families and in 268 male case and 326 male control subjects were sequentially genotyped and tested for disease association. There is no evidence that variants in the *FOXP3* regions analyzed are associated with type 1 diabetes and account for the male-female bias observed in Sardinia. Our data indicate that allelic variation in or near the coding regions of the FOXP3 gene does not have a major role in the inherited susceptibility to the common form of type 1 diabetes. *Diabetes* 53: 1911–1914, 2004

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AFBAXPAT, affected family–based X-chromosome paternal association test; FOX, forkhead/winged helix transcription factor; SNP, single nucleotide polymorphism; TPOA, autoantibody to thyroid peroxidase.

It is believed that most cases of type 1 diabetes result from an autoimmune, T-cell–dependent destruction of the insulin-producing pancreatic β-cells and subsequent irreversible insulin deficiency. Autoimmune diabetes is more commonly inherited as a common multifactorial trait but can also occur in two rare monogenic disorders, APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) and IPEX, both of which are characterized by a severe autoimmune pathology of several organs and tissues. The *FOXP3* gene and the mouse orthologue *Foxp3* are members of a gene family that encode transcription factors possessing a winged helix or forkhead box ("fox") DNA-binding domain. It has been recently shown that *Foxp3* represents a key regulator of the development and function of a subset of CD4 regulatory T-cells, which express the interleukin-2 receptor CD25, and are central in the regulation of both the adaptive and innate immune system (1–4). The elucidation of the molecular bases of these rare Mendelian disorders has provided insights into the etiology of autoimmunity in humans and in mice (2,3,5–9). It is possible that common DNA polymorphisms of *FOXP3* also influence susceptibility to the common, multifactorial form of type 1 diabetes. This hypothesis was strengthened by the observation that in common type 1 diabetes in Sardinia there is a strong male bias in disease incidence, and evidence of linkage of disease to the same region of chromosome X that encodes *FOXP3* (10,11) has been observed. Furthermore, we have excluded the involvement of a Y-chromosome gene as being the cause of the observed male excess of type 1 diabetes in Sardinian patients (11).

Taken together, these observations suggest that if common variants exist that change the function or expression of FOXP3 in more subtle ways than the described rare, highly penetrant mutations, these could help explain the elevated male-to-female ratio in young-onset cases of common type 1 diabetes and its potential linkage to chromosome X. The aim of this study was, therefore, to test if common variation at *FOXP3* was associated with the common form of type 1 diabetes in Sardinia. We initially characterized the *FOXP3* region content of single nucleotide polymorphisms (SNPs) and other polymor-

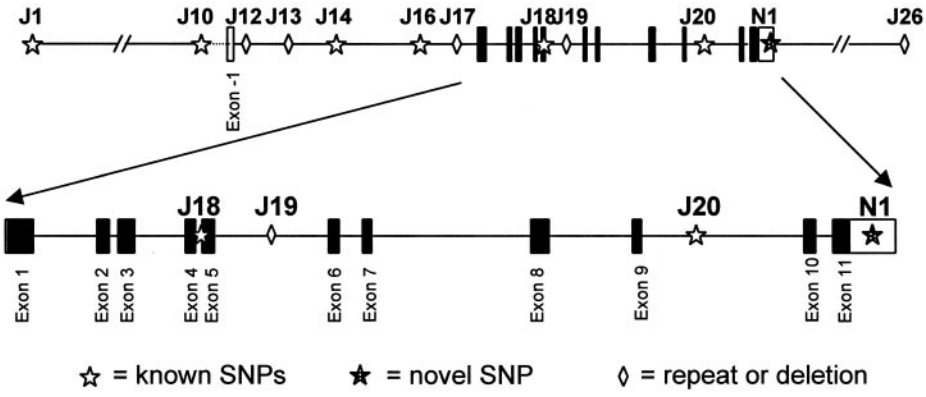

FIG. 1. Physical map of the region containing the FOXP3 gene. The 12 polymorphisms analyzed in this work are distributed in a region spanning the whole gene (from 53 kb upstream to 29 kb downstream from transcription start). The known sequence of *FOXP3* is organized into 12 exons (one of which, −1 exon, is nontranslated). All exons and introns are shown to scale, whereas the flanking regions are compressed. The positions of all polymorphisms used for analysis of association are indicated as stars (SNPs) and rumbles (microsatellites and deletions). Open and filled stars indicate known and novel SNPs, respectively.

phisms by sequencing the gene in a panel of 64 male Sardinian individuals and by supplementing and enlarging the map using polymorphisms from public databases (see RESEARCH DESIGN AND METHODS). In particular, we sequenced an overall 5.7 kb per individual, including all of the 11 coding exons and surrounding intron-exon boundaries as well as the fragments upstream of the first coding exon and downstream of exon 11, respectively, revealing no exonic or obvious splice variants. Twelve polymorphisms, including seven SNPs (one novel detected by resequencing the gene), two deletions, and three additional polymorphic markers, spanning an 82-kb *FOXP3* region were selected and genotyped in 418 Sardinian type 1 diabetic families (RESEARCH DESIGN AND METHODS and Fig. 1). In this family dataset we have, therefore, tested each marker for disease association by analyzing the maternal meioses with the transmission disequilibrium test and the paternal meioses using a test that we refer to as AFBAXPAT (affected family–based X-chromosome paternal association test) (see RESEARCH DESIGN AND METHODS). These analyses did not show any evidence of disease association at the 5% level of significance (data not shown, presented in the online appendix [available from http://diabetes.diabetesjournals.org). The intermarker linkage disequilibrium patterns indicate that there is no evidence of a break in the linkage disequilibrium between contiguous markers and, therefore, that it is unlikely that we have failed to detect a disease association due to any polymorphism in this region that was not genotyped (Fig. 2).

To further increase the statistical power of the study we selected a subset of five polymorphisms (three SNPs, one microsatellite marker, and one insertion/deletion) that extracted most of the genetic information provided by the 12 markers and tested them in an additional case-control Sardinian sample set of 268 patients and 326 healthy control subjects, consisting of only male individuals in order to also retain in the nonfamilial dataset the advantage of unequivocal phase attribution. The original families and the case-control male dataset were analyzed jointly (see RESEARCH DESIGN AND METHODS). Overall, we assembled 801 disease chromosomes and 902 control chromosomes and also found no evidence for disease association in this enlarged sample set (Table 1). For the four biallelic markers and the two more common alleles at the microsatellite J17, the power to detect association at $P = 1 \times 10^{-3}$ was >80% for a gene effect, equivalent to an odds ratio ≥1.5.

We then focused on a subset of 110 patients that, in addition to type 1 diabetes, also had celiac disease or were positive for autoantibodies to thyroid peroxidase (TPOAs), which is indicative of an immune reaction against the thyroid gland (13 type 1 diabetic patients with celiac disease, 94 TPOA positive, and 3 with both celiac disease and TPOA). We hypothesized that because FOXP3 has a pivotal role in the regulation of the immune responses it was also useful to evaluate this subgroup of type 1 diabetic patients with signs of a more general autoimmune disorder. However, in this subgroup of patients there was also no evidence of association with any of the five informative polymorphisms (Table 2).

These results are in contrast with those of Bassuny et al. (12), who studied a Japanese sample set of 199 patients and 289 control subjects and reported a weak disease association of the (GT)15 allele of the microsatellite marker located between exon −1 and exon 1 of *FOXP3* (and named J17 in our study). In our sample set, this marker did not show any evidence of association with type 1 diabetes (Tables 1 and 2). Thus, this previous claim of a

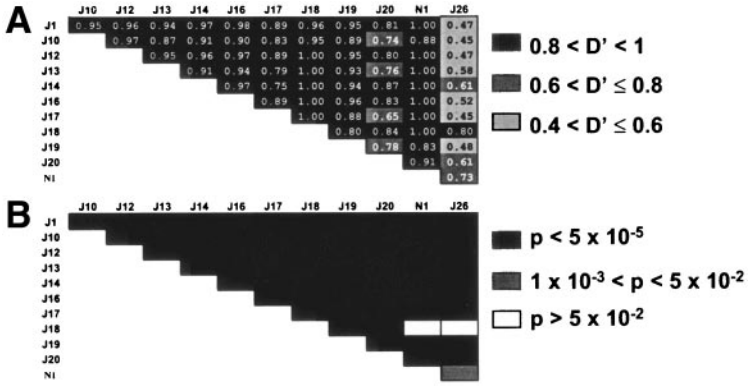

FIG. 2. Comparison of the extent and strength of linkage disequilibrium in the *FOXP3* region on the three parental chromosomes from 418 Sardinian type 1 diabetic families. Loci are listed in linear order proceeding from upstream to downstream with respect to *FOXP3*. Pairwise linkage disequilibrium estimates between each pair of loci using the Markov chain approach are shown. *A*: The distribution of intermarker D′ values are presented. Black squares correspond to a D′ value between 1 and 0.8, dark gray squares to values between 0.8 and 0.6, and light gray squares to values between 0.6 and 0.4. The individual D′ values are also specified within each cell. *B*: The corresponding *P* values are shown: black squares correspond to *P* values $<5 \times 10^{-5}$, gray squares denote *P* values between 1×10^{-3} and 5×10^{-2}, and white squares indicate *P* values $>5 \times 10^{-2}$.

TABLE 1
Association analysis of *FOXP3* with type 1 diabetes

Marker/allele	Disease chromosomes	Control chromosomes	Odds ratio (95% CI)
J13			
1	293 (39.5)	295 (36.2)	1.2 (0.9–1.4)
2	448 (60.5)	519 (63.8)	0.9 (0.7–1.1)
Total	741	814	
J14			
1	452 (56.4)	544 (60.3)	0.9 (0.7–1.0)
2	349 (43.6)	358 (39.7)	1.2 (1.0–1.4)
Total	801	902	
J16			
1	514 (65.1)	586 (66.5)	0.9 (0.8–1.1)
2	276 (34.9)	295 (33.5)	—
Total	790	881	
J17			
3	453 (58.2)	509 (60.9)	0.9 (0.7–1.1)
4	271 (34.8)	276 (33.0)	1.1 (0.9–1.3)
5	48 (6.2)	46 (5.5)	1.1 (0.7–1.7)
Other	6 (0.8)	5 (0.6)	—
Total	778	836	
J20			
1	446 (57.0)	525 (60.1)	0.9 (0.7–1.1)
2	336 (43.0)	349 (39.9)	1.1 (0.9–1.4)
Total	782	874	
J13-14-16-17-20			
(2,1,1,3,1)	309 (46.3)	350 (48.3)	0.9 (0.7–1.1)
(1,2,2,4,2)	188 (28.2)	186 (25.7)	1.1 (0.9–1.4)
(2,2,1,3,2)	35 (5.2)	35 (4.8)	1.1 (0.7–1.8)
(2,1,1,5,1)	26 (3.9)	25 (3.4)	1.1 (0.6–2.0)
(1,2,2,5,2)	18 (2.7)	16 (2.2)	1.2 (0.6–2.4)
(1,2,1,3,2)	16 (2.4)	14 (1.9)	1.2 (0.6–2.6)
(2,1,1,4,1)	15 (2.4)	14 (1.9)	1.2 (0.6–2.4)
(1,2,2,4,1)	14 (2.1)	25 (3.4)	0.6 (0.3–1.2)
(2,1,1,3,2)	9 (1.3)	18 (2.5)	0.5 (0.2–1.2)
Other	37 (5.5)	42 (5.8)	—
Total	667	725	

Data are *n* (%), unless noted otherwise. Odds ratios and 95% CIs were calculated by comparing the frequencies of the various alleles and haplotypes in the disease and control chromosomes. Only alleles and haplotypes with a frequency of ≥2% in the disease or control chromosomes are shown in this table.

positive disease association is most likely explained by random fluctuation.

We conclude that variation at, or near, *FOXP3* is not associated with type 1 diabetes in Sardinia and on its own cannot explain the male-female bias in disease incidence in this population. These results strongly support the view that variation at, or close to, *FOXP3* exons does not play a major role in the familial clustering and in general population risk of the common multifactorial form of type 1 diabetes. However, we cannot exclude that regulatory variation distant from *FOXP3* and not in linkage disequilibrium with any of the variants tested here might affect the expression of this gene and influence the disease risk.

RESEARCH DESIGN AND METHODS

In this study, we analyzed two independent sample sets: a dataset of 418 type 1 diabetic families and a case-control dataset of 268 male patients and 326 healthy male individuals, all from Sardinia. The average age at the disease onset in the 686 independent Sardinian patients was 10.4 ± 6.7 years. The 326 Sardinian control samples were from healthy adult male blood donors.

A subset of 375 type 1 diabetic patients was assayed for the presence of anti–thyroid peroxidase antibodies indicative of an autoimmune reaction against the thyroid gland. They were also assayed for the presence of anti-endomysial and anti-transglutaminase antibodies as a screening assessment for the presence of celiac disease. Patients who had anti–thyroid peroxidase antibody values >100 units/ml were considered to be positive in this study. Patients found positive for the presence of anti-endomysial and/or anti-transglutaminase antibodies were subjected to intestinal biopsy and to a standard diagnostic gluten-free/gluten trial before establishing a diagnosis of celiac disease.

FOXP3 polymorphism content. We characterized the *FOXP3* region content of SNPs and other polymorphisms, such as microsatellites and microdeletions. We resequenced the gene in a panel of 64 male individuals, 32 type 1 diabetic patients and 32 healthy control subjects: all 11 coding exons and surrounding intron-exon boundaries, as well as the regions 1,330 bp upstream of the first exon and 988 bp downstream of exon 11, comprising a total of 7.5 kb per individual DNA. To sequence these regions, we designed 13 pairs of primers for PCR fragments (reported in the online appendix). We identified two novel variants that are referred to as, respectively, N1: C>T, 172 bp 3′ from exon 11, and N2: G>A, 673 bp 3′ from exon 11. One of these novel variants, N2, was extremely rare, being present in only one control subject, and was not further typed. The N1 variant, being present in four patients and two control subjects, was instead more common and was therefore typed and tested for association with type 1 diabetes together with other 11 known polymorphisms (see below). The position and sequence of these 11 polymorphisms, which included six SNPs, three microsatellite markers, and two insertions/deletions, were obtained from public databases (accession number AF235097, http://www.ncbi.nlm.nih.gov).

Genotyping. The DNA fragments containing the six known SNPs were amplified by PCR, and the products that were dot blot analyzed using primers and the sequence-specific oligonucleotide probes are reported in the online appendix. The novel SNP was genotyped by using the MGB TaqMan technology (ordered to the Applera by the "assay by design"). The other five polymorphisms (microsatellites and deletions) were genotyped by separating fluorescently tagged PCR products on a 96-capillary sequencer (MegaBACE 1000) using the Genetic Profiler software (Amersham-Pharmacia Biotech, Buckinghamshire, U.K.). The primers used to amplify these fragments are also reported in the online appendix. Numerical values (1, 2, 3, etc.) were given to

TABLE 2
Association analysis of *FOXP3* in a subset of 110 type 1 diabetic patients with celiac disease or who were positive for TPOAs

Marker/allele	TPOA/celiac disease/type 1 diabetes disease chromosomes	Control chromosomes	Odds ratio (95% CI)
J13			
1	48 (37.2)	295 (36.2)	1.0 (0.7–1.5)
2	81 (62.8)	519 (63.8)	1.0 (0.7–1.4)
Total	129	814	
J14			
1	82 (55.4)	544 (60.3)	0.8 (0.6–1.2)
2	66 (44.6)	358 (39.7)	1.2 (0.9–1.7)
Total	148	902	
J16			
1	97 (65.5)	586 (66.5)	1.0 (0.7–1.4)
2	51 (34.5)	295 (33.5)	1.0 (0.7–1.5)
Total	148	881	
J17			
3	90 (64.3)	509 (60.9)	1.2 (0.8–1.7)
4	41 (29.3)	276 (33.0)	0.8 (0.6–1.2)
5	9 (6.4)	46 (5.5)	1.2 (0.6–2.5)
Other	—	5 (0.6)	—
Total	140	836	
J20			
1	82 (56.2)	525 (60.1)	0.9 (0.6–1.2)
2	64 (43.8)	349 (39.9)	1.2 (0.8–1.7)
Total	146	874	

Data are *n* (%), unless noted otherwise. Odds ratios and 95% CIs were calculated comparing the frequencies of the various alleles and haplotypes in the disease and control chromosomes. Only alleles and haplotypes with a frequency of ≥2% in the disease or control chromosomes are shown in this table.

microsatellite alleles starting from the allele with the lowest number of repeats.

Statistical analysis. The 12 polymorphisms of interest were typed and tested for disease association in the dataset of 418 type 1 diabetic families. To this aim, we adapted the transmission disequilibrium test to an X-linked inheritance by evaluating any departure from a random expectation of 50% in the transmission of alleles or haplotypes from heterozygous mothers to affected children (13). Because the male excess observed in type 1 diabetic Sardinian patients (10,11) is consistent with an X-linked recessive inheritance, we reasoned that paternal contribution to affected daughters could also be important for the full expression of the disease risk. In order to evaluate the paternal meioses, we developed a new test, which we refer to as AFBAXPAT. In this new test, the "disease chromosomes" are represented by the variants (alleles and haplotypes) obligatorily transmitted from the fathers to the affected daughters, whereas the "control chromosomes" are represented by the variants of the fathers having only affected male children (i.e., with no affected daughters). The disease and control alleles and haplotypes were then arranged in a 2×2 contingency table and compared using a χ^2 test under the null hypothesis of no marker association with the disease and assuming random mating and Hardy-Weinberg equilibrium.

The five most informative polymorphisms, based on evaluation of the linkage disequilibrium between variants, were also analyzed with a case-control design using a larger dataset that included both the 418 families and the case-control dataset of 268 male patients and 326 healthy male individuals. In this mixed dataset, the "disease chromosomes" are represented by the variants inherited by the affected children (only probands in families with more than one affected sibling were considered), whereas the control "population" is constructed from three sources: *1*) the variants detected in the 326 male blood donors, *2*) the variants not transmitted from the mothers to the affected child (or in the case of multiplex families, never transmitted to any affected child), and *3*) the variants detected in the fathers having only affected male children as outlined in the above-described AFBAXPAT. The disease and control allele and haplotype counts were compared using a χ^2 test under the null hypothesis of no marker association with the disease and assuming random mating and Hardy-Weinberg equilibrium. The statistical power of our sample set has been computed, considering the individual frequencies of the various alleles in the general population, based on standard epidemiological measures applied to 2×2 contingency tables.

The choice of the markers to be followed up in the case-control dataset was reached empirically by sorting the observed haplotypes and selecting the most informative variants and removing the redundant ones. The selection and definition of the relative redundancy of each marker were assisted using the intermarker linkage disequilibrium estimates and the software CZAllClust, written by C. Zavattari and available at http://mcweb.unica.it/immunogeneticslab. The linkage disequilibrium patterns between the marker loci assessed in this study were calculated on the parental chromosomes from 418 families, using a normalized disequilibrium (total D′), multiallelic extension of Lewontin's standardized measure of disequilibrium (14,15). The D′ values range from 0 to 1, with 0 reflecting perfect independence between alleles at the two loci compared and 1 reflecting complete linkage disequilibrium (14,16). The multiallelic D′ value was calculated by the program haploxt, which is available at http://archimedes.well.ox.ac.uk/pise/haploxt-simple.html. The respective *P* values were calculated using the Markov chain method described by Guo and Thompson (15) (available at http://anthropologie.unige.ch/arlequin). In all cases, 100,000 tables were explored. Unequivocal, phase-known haplotypes were established by following the cosegregation of alleles within the type 1 diabetic families or by selecting male individuals.

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