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Further Evidence for Linkage of Gilles de la Tourette Syndrome (GTS) Susceptibility Loci on Chromosomes 2p11, 8q22, and 11q23-24 in South African Afrikaners

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Utilizing DNA samples from 91 Afrikaner nuclear families with one or more affected children, five genomic regions on chromosomes 2p, 8q, 11q, 20q, and 21q that gave evidence for association with GTS in previous case-control association studies were investigated for linkage and association with GTS. Highly polymorphic markers with mean heterozygosity of 0.77 were typed and resulting genotypes evaluated using single marker transmission disequilibrium (TDT), single marker haplotype relative risk (HRR), and multi-marker "extended" TDT and HRR methods. Single marker TDT analysis showed evidence for linkage or association, with *p*-values near 0.05, for markers D2S139, GATA28F12, and D11S1377 on chromosomes 2p11, 8q22 and 11q23-24, respectively. Extended, two-locus TDT and HRR analysis provided further evidence for linkage or association on chromosome 2 with *p*-values of 0.007 and 0.025, and chromosome 8 with *p*-values of 0.059 and 0.013, respectively. These results provide important additional evidence for the location of GTS susceptibility loci.

INTRODUCTION

While the etiology of Gilles de la Tourette syndrome (GTS) —a childhood-onset neurologic disorder characterized by chronic involuntary movements (both motor and vocal tics at the time of diagnosis)—remains hidden, it has a strong genetic component [Price et al., 1985]. According to recent complex segregation analyses, the susceptibility for GTS is more complicated than previously suggested, conveyed by an additive major locus in combination with a multifactorial background [Walkup et al., 1996]. A failure to identify GTS-linked genetic loci in large, multiple affected kindreds (no LOD > 2) [Barr et al., 1999] motivated the redirection of gene-mapping efforts toward allele-sharing methods and linkage disequilibrium (LD) studies in genetically isolated populations.

Lander and Kruglyak [1995] proposed stringent guidelines for the assignment of genome-wide significance levels in linkage studies to diminish the rates of false positive results. However, in case-control LD studies, because of the danger of spurious associations due to mismatching of controls and population admixture, consistent replication may be the best evidence for a true association [Kidd, 1993].

Our previous whole-genome search for association with GTS among Afrikaners with 1,167 short tandem repeat polymorphisms, using a cross-sectional case-control strategy, DNA pooling, and follow-up individual typing of two independent samples, resulted in the identification of 15 markers at 11 chromosomal regions showing significant allele distribution differences between the two groups [Simonic et al., 1998].

For the reasons outlined above, our current study was designed as an attempt to replicate our previous results. Therefore, in this study, we collected additional independent affected individuals and their parents

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TABLE I. 31 Markers in Five Genomic Regions Selected for the Replication Study

Chr	Marker	Locus	cM	Het.	Chr	Marker	Locus	cM	Het.
2	AFM177xh4	D2S139	101.56	0.82	11	DRD2		105	0.63
2	GATA6F08	D2S440	103.16	0.68	11	Mfd316	D11S1377	120.87	0.78
2	AFMa126zb1	D2S2161	105	0.78	11	AFM331yc5	D11S1353	122.47	0.79
2	Mfd337	D2S417	106.84	0.75	11	GATA64D03	D11S4464	123	0.78
					11	AFM240ye1	D11S933	124.07	0.8
8	ATA19G07	D8S1119	101.01	0.8	11	Mfd251	D11S975	126.21	0.79
8	AFM147yb6	D8S1707	101.69	0.7					
8	AFM165yb10	D8S271	102.62	0.77	20	GATA45B10	D20S480	79.91	0.74
8	GATA8B01		103.69	0.74	20	GATA46C01	D20S1085	82.07	0.86
8	MTG8[a]		103.69	0.84	20	AFM276xh1	D20S120	83.51	0.85
8	T7-27[b]		103.69	0.43	20	GGAA11E12	D20S469	84.78	0.79
8	AFM165xh4	D8S270	103.69	0.79					
8	GATA28F12		104.33	0.52	21	GATA45C03		31.26	0.7
8	AFMa052wh1	D8S1822	107.97	0.77	21	AFMa086yf9	D21S1920	31.99	0.75
8	GATA23D12	D8S1129	110.2	0.29	21	AFMb280xd9	D21S1895	33.84	0.82
8	AFM077ya5	D8S257	111.68	0.71	21	AFM261zg1	D21S1252	35.45	0.8
8	AFM352td9	D8S559	112.42	0.72					
8	AFM333vb9	D8S1808	113.16	0.53					

[a]MTG8 3′UTR microsatellite polymorphism (11).
[b]T7-27 primers: AGCATAACATTGCTGCTAGAG and GTTACTTTGCAGATCTTTGAGC.

from the Afrikaner population. We then applied transmission disequilibrium (TDT) and haplotype relative risk (HRR) tests to genotype data generated at 5 previously determined regions of interest, to further investigate the significance of our initial findings. In three of these five regions, our previous results were confirmed ($p < 0.05$).

RESULTS

Genotypes for 4, 13, 6, 4, and 4 markers (Table I), mapping to regions on chromosomes 2, 8, 11, 20, and 21, respectively, previously found to be associated with GTS [Simonic et al., 1998], were tested for linkage and/or association in 91 independent families. Single and multiple marker TDT tests were performed using all 31 markers on these 5 chromosomes. Results of single and multiple marker TDT and HRR analyses, which either produced significant results in the original case-control study [Simonic et al., 1998] or significant results in the current study, are summarized in Table II. All non-reported p-values were greater than 0.1.

Although no single marker HRR test indicated significant association, single marker TDT analysis showed evidence for linkage or association, producing p-values of 0.039, 0.056 and 0.022, for markers D2S139, GATA28F12, and D11S1377 on chromosomes 2, 8 and 11, respectively. Two-marker extended TDT and HRR analysis provided further evidence for linkage or association on chromosomes 2, producing p-values of 0.007 and 0.025, and on chromosome 8, producing p-values of 0.059 and 0.013, respectively. Moreover, three-marker HRR analysis on chromosome 8 also showed significant association, producing a p-value of 0.011. No evidence, however, was found for linkage or association with markers on chromosomes 20 and 21.

TABLE II. Results Summary From TDT and HRR Analyses

	Original Case-Control Study (5)			Follow Up Familial TDT and HRR Study			
					Extended Haplotypes		
Chr	Locus	cM from p-tel	p	1-Locus TDT p	2-Locus TDT p	2-Locus HRR p	3-Locus HRR p
2	D2S139	101.56		**0.039**	**0.007**	**0.025**	
	D2S440	103.16	0.002	0.734			
	D2S417	106.84		0.160			
8	D8S1119	101.01	0.01	0.349			
	T7-27	103.69		0.835			**0.011**
	D8S270	103.69		0.823	**0.059**	**0.013**	
	GATA28F12	104.33		**0.056**			
	D8S257	111.68	0.01	0.638			
11	D11S1377	120.87	< 0.000001	**0.022**	0.109	0.108	
	D11S1353	122.47		0.135			
	D11S933	124.07	0.0009	0.535			
20	D20S1085	82.07	0.0001	0.240			
	D20S469	84.78	0.1	0.411			
21	GATA45C03	31.26	0.0004	0.221			
	D21S1252	35.45	0.000008	0.279			

DISCUSSION

In a genome scan using DNA samples from GTS patients and unaffected control subjects from the South African Afrikaner population, we previously identified a number of regions significantly associated with GTS [Simonic et al., 1998]. However, due to possible effects of admixture, heterogeneity, or stratification, tests for disease-marker association within nuclear families via TDT or HRR, are generally considered more rigorous than cross-sectional case-control studies. Therefore, the current study recruited Afrikaner families with affected children suitable for application of TDT and HRR techniques, in an attempt to confirm our original case-control results. Although we used a relatively small sample, we were still able to confirm 3 out of 5 chromosomal regions previously identified. Regions centered at about 2p11, 8q22, and 11q23-24 are therefore strengthened as locations for genes which influence GTS in the Afrikaner population.

During the preparation of this manuscript, two important papers published results for GTS linkage scans. The first study, by The Tourette Syndrome Association International Consortium for Genetics [1999], performed a complete genome screen in 76 families with a total of 110 sib pairs affected by Gilles de la Tourette syndrome. In this affected sib pair study, although no results reached the accepted level for significant ($MLS_{PT} > 3.93$) nor suggestive ($MLS_{PT} > 2.45$) linkage [Lander and Kruglyak, 1995; Nyholt, 2000], regions on chromosomes 4 (D4S1625) and 8 (D8S1106) produced two-point "nonparametric" MLS values above 2.0. Additional regions on chromosomes 1 (D1S1728), 4 (D4S403, D4S2623, D4S1644), 6 (D6S1053), 8 (D8S1130, D8S1145, D8S136), 10 (D10S1213), 11 (D11S912), 14 (D14S592), and 17 (D17S1298) produced two-point MLS values between 1.0 and 2.0. Multipoint MLS values above 2.0 were also obtained on chromosome 4 (including markers D4S1644 and D4S1625) and two adjacent regions on chromosome 8 (bounded by markers D8S1106–D8S1145 and at D8S136). Additional regions with multipoint MLS values above 1.0 were observed on chromosomes 1, 10, 13, and 19.

The second study, by Mérette et al. [2000], performed model-based linkage analysis in a large French Canadian family (127 members), 20 of whom were definitely affected by GTS and 20 others presenting with related tic disorders. The Mérette study, which tested the 24 markers we previously obtained evidence for LD (Tables 1 and 2 in Simonic et al. [1998]), obtained a maximum two-point LOD score of 2.4 at D11S1377 and a three-point (D11S1377–D11S933) LOD of 3.24 (10 cM centromeric to D11S1377). The study also obtained a maximum two-point LOD score of 3.2 at D13S788, however the three-point (D13S788–D13S1325) maximum LOD was only 0.0. Therefore, although regions on chromosomes 11 and 13 both produced LODs exceeding their significant linkage threshold of 3.18 (i.e., they didn't perform a genome-wide scan but tested three separate penetrance models) the results for chromosome 13 remains ambiguous (as noted by the authors). Also noted by the authors, the chromosome 11 peak is particularly interesting in that D11S933 is only 7 cM from The Tourette Syndrome Association International Consortium for Genetics [1999] implicated locus D11S912, and D11S1377 is the marker we previously obtained our most significant LD with [Simonic et al., 1998]. Therefore, the present study's significant TDT result for D11S1377 provides further and compelling evidence for the presence of a susceptibility gene near this locus. Furthermore, these results are consistent with those obtained by Gordon et al. [2000] who investigated background LD in the Afrikaner population and found evidence for it stretching across a distance of over 5 cM. This high level of background LD helps explain why some markers several cM *apart* and *from* our initial and other study's findings, show significant linkage and/or association and subsequently show why the Afrikaner population is particularly well suited for LD mapping. The sample size required to replicate previous significant results, is an important issue in any confirmation study. In complex diseases, where multiple susceptibility genes contribute towards the trait, some genes will by chance be most prevalent in a given sample, and these are the loci that will most likely be detected in an initial search. However, in a replication study focusing on specific loci previously identified, it is unlikely that these will again be most frequent in the new data set [Lernmark and Ott, 1998]. For example, Suarez et al. [1994] demonstrated, using a simple model where each locus was of equal effect and the same criteria for significance was used at the two stages, that in general, if N is the number of loci, the median sample size for replication is on the order of $N-1$ times that required for the initial linkage detection. Hence, the current study's inability to confirm our previous significant results on chromosomes 20 and 21 may reflect the relatively small size of our current sample.

In addition to sample size, there are several other potential reasons why we could not confirm the remaining two regions on chromosomes 20 and 21. Instead of true association with disease-predisposing alleles, the results for these chromosomes could be due to factors such as population admixture, slight differences in ancestry between cases and controls, and multiple founding alleles. Alternatively, the original findings could be true, but replication failed because we selected markers, which had particularly high mutation rates or had common alleles associated with the disease allele.

It is worth noting that 17 of the 91 families used in the present study, also contributed individuals to the case population used in our original case-control association studies [Simonic et al., 1998]. Therefore, although strictly speaking the two samples are not completely independent, because the present study utilizes parental genotypes in tests for linkage and/or LD, we view the tests and therefore the studies, as being independent. Furthermore, is also worth noting that the TDT and HRR tests are not necessarily expected to produce the same results. Differences are due to: i) the TDT test uses all affected siblings and is

thus essentially a test for linkage (in the *presence* of LD), while the HRR test uses only the first affected child in a nuclear family and is thus strictly a test for LD, subsequently there is a difference in the sample size used for each test; and ii) even when both tests are performed on families with just one affected child (i.e., singleton), the HRR test utilizes homozygote parental genotypes, while the TDT does not.

Our results continue to indicate that the genetics of GTS are complex even within the isolated Afrikaner population. Susceptibility genes appear to be distributed among several chromosomal sites, and more than one founder haplotype at each of these sites is likely.

Nonetheless, our findings provide an important addition to the molecular genetics of GTS and will aid in the development of future studies. For example, we report evidence for linkage or association with GTS by TDT and HRR analysis, at marker locus GATA28F12 on chromosome 8q. GATA28F12 maps just proximal to D8S257 (Table II), which has previously been associated with GTS by us [Simonic et al., 1998], and some support for linkage was reported by Leppert et al. [1996]. GATA28F12 is also close to a chromosome breakpoint (unpublished results) in a family with GTS co-segregating with a balanced translocation t(1,8) (q21.1, q22.1) as described by Devor and Magee [1999].

In summary, the results from this study represent significant ($p < 0.05$) replication and provide favorable evidence for the presence of susceptibility loci in several genomic regions. In particular, chromosomes 2p11, 8q22, and 11q23-24 as candidate regions for GTS susceptibility deserve further study. We plan to continue these investigations either by increasing our current sample size; by focusing on historical subgroups of Afrikaners and thus escalating the chances for detection of shared haplotypes; and ultimately by screening of candidate genes in these regions.

MATERIALS AND METHODS

Patients

Eighty five randomly selected GTS individuals from 74 independent families (64 singletons, 9 pairs and 1 affected trio), not participating in our previous study, were invited together with their parents for interviews and blood sample collection. They were identified according to DMS IV criteria as individuals with GTS during previous visits to the Tourette Syndrome Clinic in Pretoria and interviewed for the second time using the criteria of the Tourette Syndrome Association Genetic Consortium [TSA Classification Study Group, 1993]. For 19 individuals (including two pairs of siblings), only one parent was available for the study. In addition, parental and affected individual blood samples were collected for 22 GTS individuals from 17 independent families (12 singletons and 5 affected pairs), whose DNA samples were used in our initial genome screen. Our combined material thus consisted of 91 families with 107 GTS individuals, 88 with both parental DNA samples available, and 19 with one parental DNA only. All families spoke Afrikaans and had Afrikaner family names on both maternal and paternal sides.

Polymorphisms

In total, 31 short tandem-repeat polymorphisms (STRPs) mapping to 5 genomic regions were selected for this replication study (Table I). The main criteria for marker selection were map position (http://www.marshmed.org/genetics), spacing (~1.5 cM separation on sex-averaged genetic map), and heterozygosity (mean 0.77) [Broman et al., 1998]. Primer pairs were mostly obtained from Research Genetics. Primers for CA dinucleotide repeat marker T7-27 were designed from the partial sequence of BAC clone 127D17, which contains the MTG8 gene. Allele sizes for the markers were determined using known genotypes of two CEPH individuals (133101 and 133102).

PCR

Amplification of STRPs was performed in 96 well microtiter plates with 50 ng of DNA in a 5 μl volume containing 3 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 0.01% (w/v) gelatin; 200 μM each dGTP, dCTP, and dTTP; 2.5 μM dATP; 0.35 μCi α[33P]-dATP (NEN Du Pont; 800 Ci/mmol, 10 μCi/μl); 10 ng (~3 pmol) each PCR primer; and 0.3 U Taq polymerase (Sigma). Samples were subjected to 29 cycles consisting of 30 sec at 94°C, 75 sec at 55°C, and 30 sec at 72°C, with a final 6 min at 72°C after the last cycle. All markers were amplified individually. All genotyping was performed in duplicate to minimize errors. PCR products were denatured by heating for 10 min at 95°C prior to loading (0.5 μl) onto vertical 6.5% polyacrylamide, 7.7 M urea DNA sequencing gels and running at 70 W constant power for ~2 hours. Gels were dried on filter paper and exposed on X-ray film. Allele sizes were determined using known genotypes of two CEPH individuals 133101 and 133102 and Caucasian pooled DNA sample (Research Genetics).

Statistical Analysis

Several different statistics were used to evaluate marker genotypes (calculation of *p*-values is described in paragraph 4 below):

1. Single marker transmission disequilibrium test (TDT) [Spielman and Ewens, 1996], based on a statistical comparison of the frequencies of transmissions and nontransmissions of marker alleles from heterozygous parents to affected offspring. The distribution of transmitted versus nontransmitted alleles were compared using the T_m (χ^2) TDT statistic [Bickeböller and Clergot-Darpoux, 1995].
2. Single marker haplotype relative risk (HRR) method, first proposed by Falk and Rubinstein [1987], uses alleles of the parents (both homozygous and heterozygous) which are not transmitted to an affected child to create a so called *pseudocontrol* [Schaid, 1998] (for the few parents with multiple affected offspring, only

their first affected child is used). The control alleles are assigned in relation to the number of typed parents, using the following rules: (i) if both parents are typed then the two nontransmitted alleles in the parents of the affected child are included in the control sample; (ii) if only one parent is typed and there is an unambiguous solution (i.e., parent and child have different genotypes) then the one remaining (nontransmitted) allele is included in the control sample; and (iii) if neither parent is typed, or if only one parent is typed and has the same genotype as the affected child, in which case the probability of the transmitted and nontransmitted alleles are ambiguous, then no allele(s) is included in the control sample. The association of disease with marker alleles were assessed by a traditional case-control χ^2statistic.

3. Multipoint TDT and HRR methods were devised to increase meiotic informativeness within families. Multi-marker haplotypes were built across two or three contiguous markers (i.e., 1-2, 2-3, 3-4 and 1-2-3, 2-3-4, 3-4-5, etc.). A transmitted haplotype consisted of alleles at contiguous loci, which could be unambiguously determined to be transmitted by a parent to an affected offspring, and non-transmitted haplotypes were defined analogously. The only case in which transmitted haplotypes are ambiguous occurs when both of the typed parents plus the affected child are all heterozygous for the same alleles at the same locus, in which case these individuals were excluded from the analysis. The frequencies of these transmitted and nontransmitted haplotypes were then statistically tested for linkage and association using the same methods used for the single marker tests.
4. Because the high number of allele and haplotype combinations produce large, sparse contingency tables, significance for all TDT and HRR tests was determined by exact methods. Associated p-values were approximated by Monte Carlo simulation as implemented in the StatXact3 program (CYTEL Software Corp., Cambridge, MA) with 10,000 iterations. Also, because this is a replication analysis focusing on previously identified genomic regions, p-values are reported without correction for multiple testing and the significance threshold was set at $p = 0.05$.

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