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Identification of Genetic Markers Associated with Gilles de la Tourette Syndrome in an Afrikaner Population

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Summary

Because gene-mapping efforts, using large kindreds and parametric methods of analysis, for the neurologic disorder Tourette syndrome have failed, efforts are being redirected toward association studies in young, genetically isolated populations. The availability of dense marker maps makes it feasible to search for association throughout the entire genome. We report the results of such a genome scan using DNA samples from Tourette patients and unaffected control subjects from the South African Afrikaner population. To optimize mapping efficiency, we chose a two-step strategy. First, we screened pools of DNA samples from both affected and control individuals, using a dense collection of 1,167 short tandem-repeat polymorphisms distributed throughout the genome. Second, we typed those markers displaying evidence of allele frequency–distribution shifts, along with additional tightly linked markers, using DNA from each affected and unaffected individual. To reduce false positives, we tested two independent groups of case and control subjects. Strongest evidence for association (*P* values 10^{-2} to 10^{-5}) were obtained for markers within chromosomal regions encompassing D2S1790 near the chromosome 2 centromere, D6S477 on distal 6p, D8S257 on 8q, D11S933 on 11q, D14S1003 on proximal 14q, D20S1085 on distal 20q, and D21S1252 on 21q.

Introduction

Gilles de la Tourette syndrome (MIM 137580) is a childhood-onset neurologic disorder characterized by chronic, involuntary motor and vocal tics. Both twin and family studies have indicated a high degree of heritability of Tourette syndrome, especially when individuals with chronic motor tics are considered affected (Patel 1996). Concordance rates, among MZ twins, of 53% and 56% for full-blown Tourette syndrome and 77% and 94% for Tourette syndrome plus chronic motor tics have been reported (Price et al. 1985; Hyde et al. 1992). Concordance rates for DZ twins were 8% for Tourette syndrome alone and 23% for Tourette syndrome plus chronic motor tics (Price et al. 1985). Earlier segregation analyses indicated an autosomal dominant mode of inheritance, with reduced penetrance (Comings et al. 1984; Pauls and Leckman 1986; Eapen et al. 1993). More-recent studies, however, have indicated a more complex mode of inheritance (Hasstedt et al. 1995; Walkup et al. 1996).

Tourette syndrome has been reported to cosegregate with several neuropsychiatric disorders. A connection between Tourette syndrome and obsessive-compulsive disorder is generally accepted (Pauls et al. 1991, 1995). Connections between Tourette syndrome and attention-deficit disorder and between other disorders such as alcoholism, panic attacks, and conduct disorders are controversial (Comings and Comings 1988; Pauls et al. 1988; Comings 1994).

A number of large Tourette kindreds have been identified (Kurlan et al. 1986; Robertson and Trimble 1991; McMahon et al. 1992; Hasstedt et al. 1995; Heutink et al. 1995). Several of these kindreds have undergone whole-genome polymorphism screening. Parametric linkage analysis of the resulting data, under the assumption of autosomal dominant inheritance, failed to locate genes (Pakstis et al. 1991; Wilkie et al. 1992; van de Wetering and Heutink 1993). Reasons for the failure are unknown but may be related to the wide range of disease severity within Tourette kindreds and/or to misspecification of the mode of inheritance. Associations between three dopamine receptor genes and Tourette syndrome have been reported (Comings et al. 1993; Nöthen et al. 1994; Grice et al. 1996), but replication has not been achieved (Hebebrand et al. 1997).

The South African Afrikaner population arose largely

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from a small group of European, primarily Dutch, immigrants who began settling in Cape Town in 1652 (Torrington et al. 1984; Jenkins 1990; see also Government Communication and Information System [South Africa]). By 1701, there were 1,265 Europeans at the Cape. In the 18th century, European immigration to South Africa was small. In the first half of the 19th century, particularly in the 1830s, the Afrikaners began the "Great Treks," a series of migrations of Europeans away from the coastal areas, to establish farming communities in the interior of South Africa. The small number of European founders of the Afrikaner population at Cape Town, the lack of large waves of European immigration, the Great Treks, and the relatively rapid increase in the Afrikaner population, today at ~3 million, all have contributed to the genetic isolation and relative homogeneity of this population. Common mutations and/or marker haplotypes among Afrikaners have been established for several heritable disorders, including porphyria variegata, keratolytic winter erythema, hypercholesterolemia, and progressive familial heart block (Jenkins 1990, 1996; Meissner et al. 1996; Warnich et al. 1996; Starfield et al. 1997; Groenewald et al. 1998).

We report here our efforts to map Tourette syndrome genes, using Afrikaner patients. Starting with a whole-genome screen of >1,000 polymorphisms, we have identified several markers that show significant differences in allele frequency distributions between affected and control individuals.

Subjects, Material, and Methods

Patients

Probands were selected at random, on consecutive clinic days, from a group of existing patients at the Tourette Syndrome Clinic in Pretoria, South Africa. All subjects were previously identified as Tourette syndrome patients according to criteria of the *Diagnostic and Statistical Manual of Mental Disorders*. Motor and vocal tics were in the moderately severe to severe range in all affected individuals. Tics started at <18 years, occurred for $\geqslant$1 year, and were not absent for any periods >3 mo. Prior to their recruitment, probands and available family members were evaluated personally, by two educational psychologists, for history of tics and presence and severity of comorbid problems. The following diagnostic tools were used: a self-report form designed by the Tourette Syndrome Association Genetic Consortium (January 1995 version), the Yale Global Tic Severity Scale (Leckman et al. 1989), observation/examination methods for the purpose of excluding other movement or neurological disorders and for confirming observable tics typical of those associated with Tourette syndrome, and the *Stony Brook Psychiatric Exclusion Checklist* (Gadow and Sprafkin 1998). English language versions of all forms were used, as most Afrikaners, including all patients, control subjects, and evaluators, were bilingual.

Forty Tourette syndrome patients were recruited in the initial phase of the study (first group). For the follow-up investigations, an additional 60 unrelated patients agreed to participate (second group). All study subjects spoke Afrikaans and had Afrikaner family names but were not known to belong to any Afrikaner subgroup. Individuals of ascertainable English descent were excluded. The first and second groups of patients (and control subjects) were selected from the same population base. All blood samples were coded and made anonymous prior to DNA isolation. Only the information on age, sex, tics severity range, and comorbid behavioral problems remained attached to each sample. The study was approved by the South African Medical Research Council Review Board.

Control Subjects

The Afrikaner individuals ($n = 96$) included in the control group came from two sources: clinic personnel and medical students. Like the patients, all control subjects spoke Afrikaans and had Afrikaner family names. Control subjects were required to complete the same self-report form used by the patients and, in addition, were personally evaluated by one of us (G.S.G.) for the presence of chronic motor and vocal tics. Their blood samples were coded and made anonymous prior to DNA isolation.

Primers

Primer pairs for the detection of short tandem-repeat polymorphisms (STRPs) were obtained from Research Genetics. Approximately 40% of the markers were tri- and tetranucleotide-repeat polymorphisms developed within the Cooperative Human Linkage Center, and nearly all of the remaining 60% were Généthon dinucleotide-repeat polymorphisms. We determined allele sizes, using known genotypes of three CEPH individuals: 133101, 133102, and 134702. Marker spacing was determined and close flanking markers selected by use of comprehensive sex-averaged genetic maps produced by the program CRI-MAP (Lander and Green 1987; Broman et al. [in press]; see also Center for Medical Genetics).

Sampling and DNA Pooling

Genomic DNA was isolated from individual blood samples by standard methods (Sambrook et al. 1989). DNA concentrations were measured by spectrophotometric readings at 260 nm. Pools of template DNA were prepared by combining 1 μg of DNA from each of 20 individuals from the first group of subjects. Two non-overlapping pools were prepared from the affected in-

dividuals (A1 and A2) and from the controls (C1 and C2). The pooled DNA was diluted to a final concentration of 20 ng/μl.

PCR

Amplification of STRP markers was performed in 96-well microtiter plates with 45 ng of either pooled DNA or individual DNA in a 10-μl volume containing 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 0.01% (w/v) gelatin; 200 μM each dGTP, dATP, and dTTP; 2.5 μM dCTP; 0.35 μCi $\alpha[^{32}P]$-dCTP (NEN Du Pont; 800 Ci/mmol, 10 μCi/μl); 10 ng (~3 pmol) each PCR primer; and 0.3 U AmpliTaq polymerase (Boehringer Mannheim). In most cases, two markers were amplified simultaneously. Samples were subjected to 27 cycles consisting of 30 sec at 94°, 75 sec at 55°, and 15 sec at 72°, with a final 6 min at 72° after the last cycle. PCR products were denatured by adding formamide and by heating for 10 min at 95° prior to loading (1 μl) onto vertical 6.5% polyacrylamide, 7.7 M urea DNA sequencing gels and running at 70 W constant power for ~3 hours. Gels were dried on filter paper and exposed on X-ray film.

Statistical Analysis

The following statistics were calculated to compare affected and control allele frequency distributions for each polymorphic marker:

"Heterogeneity" approach.—For the *m* alleles at a given marker, an *m* by 2 contingency table was formed, with columns corresponding to alleles in case and control individuals. Rows were ordered by increasing allele size, and rows containing cells with expected values <1 were pooled with neighboring rows. From a χ^2 analysis, empirical (two-sided) significance levels were obtained. This approach is expected to be powerful when several alleles occur in different frequencies in the case and control subjects.

"Single alleles" approach.—A given allele, *i,* was selected, and all other alleles were combined into a second category, *not i.* The resulting 2 by 2 table was analyzed by use of Fisher's exact test. The smallest (one-sided) *P* value was selected (and the allele identified at which it occurred) and adjusted for multiple comparisons by means of a Bonferroni correction. This approach is expected to be powerful when a single allele shows association. One-sided *P* values were used because, when the frequency of a given allele is increased in case versus control subjects, some other allele(s) must necessarily be decreased. When one allele is tested after another, each allele should thus be tested only for positive association.

"t-test" approach.—Mean allele sizes for case and control subjects were compared with use of the *t*-test. Empirical *P* values (two-sided) were calculated numerically. This approach is expected to be powerful when allele sizes are shifted in a constant direction between case and control subjects.

Results

A first group of 40 Afrikaners clearly affected with Tourette syndrome, along with 40 unaffected Afrikaner controls, were recruited. DNA was pooled from two nonoverlapping sets of 20 individuals for both affected (A1 and A2) and control (C1 and C2) subjects. Two sets of pools were chosen, to reduce the false-positive rate due to slight differences in DNA concentrations and pipetting inaccuracies. The pooled DNA, along with standard DNA from the parents of CEPH family 1331, was used as template in the PCR amplification of 1,167 STRPs distributed throughout the entire genome. Chromosomal breakdown of the STRPs is shown in table 1. Sex-averaged spacing between STRPs was 3.0 ± 3.2 cM (mean ± SD). Ninety-five percent of the intervals between markers were <9.0 cM. Fifteen intervals were >15 cM, with the very largest at 22 cM.

Visual examination of the autoradiographic images of pooled PCR products revealed 51 loci with consistent differences in allele distributions between the two affected and two control DNA pools. Results from six of these loci are displayed in figure 1. Criteria for selection of the positive loci were (1) consistency of observed differences between affected and control subjects as determined by repeat PCR and by use of two independent sets of control and affected pools, and (2) prevalence of one allele in PCR products from the affected pools (indicated by arrows in fig. 1). Amplification of the vast majority of the markers resulted in indistinguishable radiographic images between affected and control pools. For a small proportion of STRPs, differences between affected and control pools were detected for A1/C1 or A2/C2, but not for both. This may be the consequence of pooling DNA from only 20 individuals.

To evaluate the statistical significance of the observed

Table 1

Chromosomal Breakdown of Markers Used in Whole-Genome Screen

Chromosome	No. of STRPs	Chromosome	No. of STRPs
1	96	13	41
2	91	14	48
3	60	15	31
4	65	16	35
5	78	17	41
6	69	18	47
7	66	19	24
8	67	20	32
9	39	21	14
10	43	22	17
11	44	X	52
12	67		

Table 2

Loci Associated with Tourette Syndrome in First and Second Groups of Affecteds

	First Group of Affecteds			Second Group of Affecteds			First and Second Groups Combined
Locus	*P* (het)	*P* (single alleles)	*P* (*t*-test)	*P* (het)	*P* (single alleles)	*P* (*t*-test)	*P* (het)
D1S485	.02	.005	.12	.25	.24	.22	.02
D1S1665	.01	.001	.04	.12	.06	.45	.008
D2S1391	.04	.16	.41	.14	.10	.29	.03
D2S1790	.01	.04	.03	.006	.01	.86	.0006
D4S1551	.01	.02	.12	.42	.64	.34	.03
D5S666	.03	.02	.07	.19	.62	.92	.03
D6S477	.005	.06	.08	.02	.04	.41	.0009
D6S470	.0006	.02	.001	.43	.21	.13	.004
D8S1119	.03	.06	.66	.09	.05	.82	.01
D11S933	.05	.006	.75	.002	.009	.38	.0009
D12S327	.001	.10	.04	.38	.30	.63	.006
D13S788	.005	.16	.50	.56	.43	.95	.02
D20S1085	.03	.03	.65	.0005	.0004	.76	.0001
D21S1252	.00001	.006	.42	.07	.12	.36	.000008
D21S1435	.04	.05	.03	.46	.17	.24	.09

Note.—het = heterogeneity. *P* values indicate the probability of obtaining a difference as large or larger than the one observed if there is no association between disease and marker. Values were calculated by means of three different approaches, as described in the Subjects, Material, and Methods section.

differences in pooled PCR products, we subjected the 51 putative positive markers to individual typing, using the same group of 40 affected and 40 control DNA samples used for the initial genomic screen. We then compared the resulting pairs of allele frequency distributions, using three different statistical approaches (see the Subjects, Material, and Methods section). For 15 of the 51 loci, at least one of the calculated statistics showed significance at the .05 level (table 2). For the remaining 36 markers, apparent differences between affected and control subjects were not confirmed at the .05 level.

In an attempt to reproduce these initial results, a second group of unrelated Afrikaners (60 affected and 56 control individuals) were recruited, and their DNA was typed individually with the 15 positive markers from the first phase of the study. Results of the typings for the second group are also listed in table 2. For the majority of loci, evidence for association was not confirmed. However, in four instances (D2S1790, D6S477, D11S933, and D20S1085), the *P* values for the second group also reached significance and were even lower than those for the first group. For all 15 markers, *P* (heterogeneity) values from the first and second groups were then combined by summing χ^2 and df values (table 2). This approach of combining results was chosen as a safeguard, to eliminate potential effects of sizing alleles differently in the two samples. Combining results increased support for association in nearly every case relative to the results from either the first or second group of samples.

To extend these results, we typed a number of markers closely linked to positive markers from the whole-genome scan, using both groups of samples jointly. Markers at several of these loci gave significant results, including D2S440 adjacent to D2S1790, D8S257 and D8S1132 close to D8S1119, D11S1377 adjacent to D11S933, D20S468 and D20S469, both very close to D20S1085, and GATA45C03 near D21S1252 (table 3). Results for D11S1377 were particularly impressive. In addition, two tightly linked markers on chromosome 14 also produced significant results (table 3). The marker at locus D14S742 was identified in the first stage of the study but did not quite yield *P* values < 0.05 for the first set of samples. No additional markers with significant allele distribution differences were identified from chromosomes 1, 6, 11, 12, or 13. Allele frequency distributions for D11S1377 and GATA45C03 are displayed in table 4. Note that, for each marker, more than two common alleles were enriched in the affected subjects compared with control subjects.

Discussion

The primary goal of this study was to identify loci associated with Gilles de la Tourette syndrome in the Afrikaner population, using the straightforward comparison of polymorphism allele frequency distributions between severely affected probands and unaffected control subjects. We believe we have accomplished this goal for the markers at the loci listed in tables 2 and 3.

Several groups have identified linkage disequilibrium over broad chromosomal intervals in isolated popula-

tions. Disequilibrium has readily been detected over intervals ranging up to 15 cM in the Finnish population (Peltonen and Uusitalo 1997), which is likely to be considerably older than the Afrikaners. Among populations thought to be similar in age to the Afrikaners (~12 generations), Houwen et al. (1994) identified disequilibrium over a 19-cM interval in patients with intrahepatic cholestasis, from an isolated Netherlands fishing village, and Puffenberger et al. (1994) found disequilibrium over at least 10 cM among American Mennonites with Hirschsprung disease. Among Afrikaners, Starfield et al. (1997) detected disequilibrium over 10 cM for keratolytic winter erythema, and Groenewald et al. (1998) recently reported shared haplotypes extending over ~17 cM for porphyria variegata. Therefore, our use of a highly informative marker density of 3.0 cM gave us a good chance of successfully detecting association among Afrikaner Tourette patients.

Table 3

Additional Loci in Selected Chromosomal Regions Associated with Tourette Syndrome

	P Value for Approach		
Locus	Heterogeneity	Single Alleles	*t*-test
D2S440	.002	.001	.54
D8S257	.01	.004	.74
D8S1132	.05	.10	.33
D11S1377	$<10^{-6}$	.0005	.01
D14S742	.06	.02	.03
D14S1003	.002	.003	.00003
D20S468	.05	.06	.005
D20S469	.10	.06	.66
GATA45C03 (chromosome 21)	.0004	.004	.04

Note.—Results were obtained with DNA from affected and control individuals in both first and second groups. See note for table 2.

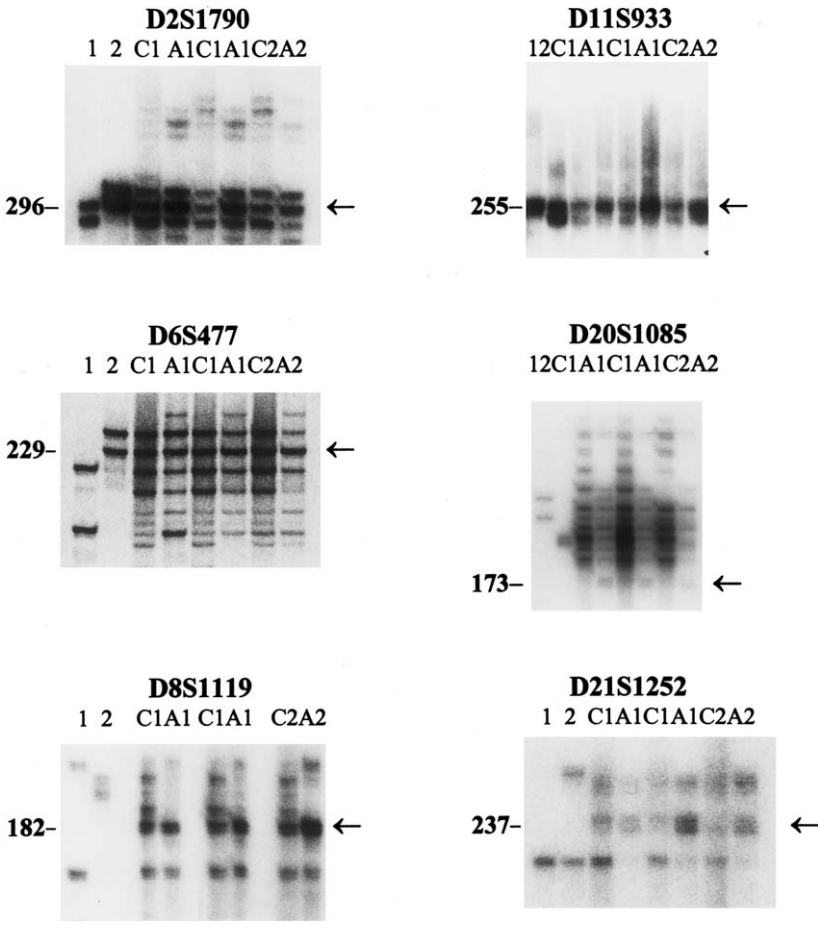

Figure 1 Electrophoretic profiles for markers that demonstrated consistent allele frequency distribution differences between affected and control subjects in the first group. Segments of autoradiographs from polyacrylamide gels are displayed for six STRPs at the indicated loci. The DNA templates used to generate the amplified DNA fragments were in the same order for each marker: standard DNA from CEPH family parents 133101 (1) and 133102 (2), DNA pool from set 1 of unaffected control subjects (C1), DNA pool from set 1 of affected subjects (A1), independent amplification of pools C1 and A1, DNA pool from set 2 of unaffected control subjects (C2), and DNA pool from set 2 of affected subjects (A2). Arrows mark alleles enriched in the affected subjects. Sizes (in nucleotides) for the enriched alleles are listed on the left.

Table 4

Allele Frequency Distributions for Two Markers with Especially Low *P* Values

Mfd316 at D11S1377			GATA45C03 (Chromosome 21)		
Allele	Affected	Control	Allele	Affected	Control
124	1	0	266	0	3
128	4	0	268	62	66
130	4	7	286	1	0
132	2	6	288	10	5
134	45	20	289	6	5
136	62	42	291	32	47
138	22	52	302	1	3
140	6	19	305	4	14
142	16	5	308	15	16
144	4	9	310	28	19
146	8	6	312	14	1
148	0	6	314	3	0
			318	0	1

Note.—Results were obtained with DNA from affected and control individuals in both first and second groups.

The use of DNA pools in the whole-genome screen dramatically reduced the amount of required laboratory work. Allele frequency distributions obtained from pooled DNA templates have been found to match, reasonably well, those determined by typing individuals (Pacek et al. 1993; Graff et al. 1997). Pooling approaches are rapidly becoming standard for the mapping of rare recessive disorders within isolated populations (Sheffield et al. 1994; Peltonen and Uusitalo 1997). Although careful scanning of the electrophoretic profiles of the amplified fragments might be ideal (Barcellos et al. 1997; Graff et al. 1997), visual selection of markers with differences in allele frequency distributions is efficient and has worked well for recessive disorders. The fact that only 15 of 51 loci that appeared positive visually were confirmed by typing individuals indicated that we were generous in our selection of candidate loci and that, although false-negative loci cannot be completely ruled out, true-positive loci were not easily missed.

Analysis of the amplified fragments from the DNA pools gave no indication that a single predominant Tourette gene exists among the Afrikaners or that Tourette genes were introduced into the Afrikaner population by a single founder. Although specific alleles at various loci were clearly enriched in affected versus control subjects, no single predominant alleles were found at any loci (see, for example, table 4). Therefore, even in the isolated Afrikaner population, several founding Tourette alleles at several loci likely exist.

Since no one yet knows how many Tourette genes exist, how they interact, how many Afrikaner founders introduced Tourette genes into this population, or when the genes might have been introduced, it is impossible to project how many patients would be required to detect association and what *P* values would be significant (Kruglyak 1997). It is likewise impossible to completely rule out subtle, undetected population differences between the patient and control groups. Nevertheless, we feel that the *P* values displayed in tables 2 and 3 are very promising. Given 1,167 markers tested, and on the basis of the conservative Bonferroni correction for multiple testing, we would by chance expect 0.1 results significant at the 10^{-4} level. In fact, we found four such markers on chromosomes 11, 14, 20, and 21. Our use of repeat PCR (testing pools A1 and C1 twice, as shown in the figure) and separate pools of affected and control individuals (pools A1, A2, C1, and C2), the confirmation of results using an entire separate group of Tourette patients, and the typing of additional STRPs tightly linked to the original positives were all designed to reduce the possibility of false positives.

We assert that the only practical route to isolation of Afrikaner Tourette genes is to pursue loci that give strong evidence for association and that eventually are confirmed by independent studies. In this regard, Leppert and McMahon, in their whole-genome–polymorphism screen of unilineal branches of a very large Utah Tourette kindred (McMahon et al. 1992), obtained some of their strongest positive LOD scores for several markers (including D8S257) in the exact same region of chromosome 8q as that identified in this study (unpublished results). Also, Devor and Magee (in press) very recently reported a family in which individuals with Tourette syndrome or tics showed segregation with a balanced chromosome 1-8 translocation [(1:8)(q21.1;q22.1)]. D8S257 is located at or very close to 8q22.1 (Bray-Ward et al. 1996). We plan to continue our efforts among the Afrikaners by collecting parents of affected individuals, so that shared haplotypes can be identified, and by collecting Tourette families from South African Gereformeerde Church members who are themselves a subgroup of Afrikaners (Torrington et al. 1984).

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Electronic-Database Information

Accession numbers and urls for data in this article are as follows:

Center for Medical Genetics, http://www.marshmed.org/genetics (for polymorphism and genetic map information)
Cooperative Human Linkage Center, http://www.chlc.org (for polymorphism information)
Généthon, http://www.genethon.fr (for polymorphism information)
Government Communication and Information System [South Africa], http://www.gcis.gov.za/level2/history.htm (for Afrikaner history)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Tourette syndrome [MIM 137580]

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