HHS Public Access

Author manuscript

Mov Disord. Author manuscript; available in PMC 2016 September 26.

Published in final edited form as:

Mov Disord. 2015 July ; 30(8): 1115–1120. doi:10.1002/mds.26279.

GDNF Gene Is Associated With Tourette Syndrome in a Family Study

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Abstract

Background—Tourette syndrome is a disorder characterized by persistent motor and vocal tics, and frequently accompanied by the comorbidities attention deficit hyperactivity disorder and obsessive-compulsive disorder. Impaired synaptic neurotransmission has been implicated in its pathogenesis. Our aim was to investigate the association of 28 candidate genes, including genes related to synaptic neurotransmission and neurotrophic factors, with Tourette syndrome.

Methods—We genotyped 506 polymorphisms in a discovery cohort from the United States composed of 112 families and 47 unrelated singletons with Tourette syndrome (201 cases and 253 controls). Genes containing significant polymorphisms were imputed to fine-map the signal(s) to potential causal variants. Allelic analyses in Tourette syndrome cases were performed to check the role in attention deficit hyperactivity disorder and obsessive-compulsive disorder comorbidities. Target polymorphisms were further studied in a replication cohort from southern Spain composed of 37 families and three unrelated singletons (44 cases and 73 controls).

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Relevant conflicts of interest/financial disclosures: Nothing to report.
Full financial disclosures and author roles may be found in the online version of this article.

Results—The polymorphism rs3096140 in glial cell line–derived neurotrophic factor gene (*GDNF*) was significant in the discovery cohort after correction ($P = 1.5 \times 10^{-4}$). No linkage disequilibrium was found between rs3096140 and other functional variants in the gene. We selected rs3096140 as target polymorphism, and the association was confirmed in the replication cohort ($P = 0.01$). No association with any comorbidity was found.

Conclusions—As a conclusion, a common genetic variant in *GDNF* is associated with Tourette syndrome. A defect in the production of GDNF could compromise the survival of parvalbumin interneurons, thus altering the excitatory/inhibitory balance in the corticostriatal circuitry. Validation of this variant in other family cohorts is necessary.

Keywords

Tourette syndrome; synaptic neurotransmission; family-based association analysis

Tourette syndrome (TS) is a childhood-onset neuropsychiatric disorder characterized by persistent motor and vocal tics. It is frequently accompanied by other psychiatric comorbidities, mostly by attention deficit/ hyperactivity disorder (ADHD; up to 60%) and obsessive-compulsive disorder (OCD in 45%–60%). Although the tics may lessen or disappear in adulthood, these comorbid symptoms may or may not disappear during adulthood. Tourette syndrome has a population prevalence between 0.3% and 1%, with a strong familial recurrence.[1]

As with many other complex diseases, both genes and environment factors may interact to produce multifactorial inheritance. Despite many TS research studies over the last two decades, its cellular and molecular basis remains poorly understood.[2–4] Several lines of evidence have involved the cortico-striatal-thalamo-cortical circuitry in this disorder.[5–7] Both studies in humans with transcranial magnetic stimulation[8–11] and the mouse models *Dlgap3/Sapap3* and *Slitrk5* for TS/OCD[12,13] have shown evidence of altered plasticity and synaptic neurotransmission. Postmortem studies have found abnormalities in the basal ganglia, in particular a decreased number of striatal cholinergic interneurons (Chat+) as well as striatal interneurons expressing parvalbumin (PV+).[14,15] Accordingly, imaging studies showed reduced gray matter volumes in the basal ganglia,[16] with the degree of reduction correlating with symptom severity in early adulthood.[17]

The dopaminergic and glutamatergic systems play major roles in cortico-striatal-thalamo-cortical pathways through complex interactions. In fact, the dopaminergic system is altered in TS, and dopamine antagonists effectively suppress tics.[5–7] For these reasons, the dopaminergic pathway has been widely investigated, and numerous genetic studies have been performed. Nevertheless, conflicting results continue, and the association with TS of some dopamine-related candidate genes such as various dopamine receptors, monoamine oxidase-A, the dopamine transporter gene, and other related genes, is unclear.[2,4] Mounting evidence has been noted about the involvement of the glutamatergic system in TS. The gene *DLGAP3*, which encodes Sapap3, a postsynaptic scaffolding protein highly expressed in striatal glutamatergic synapses, has been associated with TS in a family study.[18] Of note, the recent genome-wide association studies (GWAS) in OCD, a condition thought to share neurobiological substrate(s) and genetic background with TS, had its peak signal within

DLGAP1, which codes for Sapap1, also from the same superfamily of proteins.[19,20] Moreover, many OCD patients benefit from glutamate-altering medications.

Neurotrophic factors promote growth, survival, and differentiation of neurons and are highly expressed in the striatum. They interact with glutamate to regulate developmental and adult neuronal plasticity, and neurotrophic factor dysregulation may alter glutamate signaling, leading to neurodegenerative processes.[21] These factors are also necessary for the survival of dopaminergic neurons in the substantia nigra.[22] Of relevance among these factors are the brain-derived factor (BDNF) and the glial cell line-derived neurotrophic factor (GDNF). The *BDNF* gene has been related to OCD,[23] and the *GDNF* gene is located within a region of chromosome 5 that has been repeatedly associated with TS through linkage analyses.[24,25] Interestingly, *GDNF* is expressed by both striatal PV+ gamma-aminobutyric acid (GABA)-ergic and cholinergic interneurons,[26] cellular subtypes that, as commented before, are affected in TS.

Herein, we investigate a variety of genes involved in dopaminergic and glutamatergic neurotransmission, neurotrophic factors, and genes related to other pathways in a family-based sample of TS. We hypothesized that the alterations seen in synaptic neurotransmission in TS might have a genetic origin, and that these risk factors may be inherently involved in the pathogenesis of the disease. To test this hypothesis, we selected a set of 28 genes covering: (1) the glutamatergic system via *N*-methyl-D-aspartate receptors (*GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A, GRIN3B*), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (*GRIA1-4*), metabotropic glutamate receptors (*GRM1, GRM5*), and two genes encoding proteins involved in the regulation and trafficking of calcium channels at glutamatergic synapses (*CACNG2, CAMK2A*); (2) the dopaminergic system via the dopamine receptors (*DRD1-5*) and the dopamine transporter SLC6A3; (3) monoamine oxidases; (4) neurotrophic factors (*BDNF, GDNF*); (5) and three other genes that have been associated with several neurological and psychiatric diseases: the catechol-O-methyltransferase (*COMT*), the cannabinoid receptor 1 (*CNR1*), and the neurotrophic tyrosine receptor kinase 2 (*NTRK2*).

Methods

Study Criteria and Participants

In this study, we investigated 28 candidate genes in a discovery cohort from the United States,[27] and significant single-nucleotide polymorphisms (SNPs) were assessed in a replication cohort from southern Spain.

The discovery cohort was composed of 112 nuclear families with at least one TS proband and 47 unrelated singletons with TS, totaling 201 cases (70% males; mean age 21 ± 14 y, and mean age at onset 6 ± 3 y), and 253 unaffected family members as controls (46% males; mean age 38 ± 16 y). Families included 37 complete trios, 50 complete nuclear families with affected/discordant siblings, and 25 incomplete nuclear families (only one parent available). All unrelated TS cases and 105 families were of European ancestry, and five families were mixed, one Asian and one African American. All these non-European families were at least complete trios, and none of the parents was affected, thus not affecting population

stratification. All samples were collected within the framework of the New Jersey Center for Tourette Syndrome Sharing Repository initiative (http://www.tourette-repository.org/) and were processed at the National Institute for Mental Health Center for Collaborative Genomics Research on Mental Disorders at RUCDR (RUCDR Infinite Biologics, http://www.rucdr.org) at Rutgers University, Piscataway, New Jersey, USA. All participants were evaluated and interviewed by clinicians experienced in TS, and diagnosis was based on Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision criteria. Clinical assessments included a set of standardized and well-validated tic, OCD, and ADHD rating instruments (adult self-report or parent-on-child report). Further details about the subjects' recruitment, data collection, and clinical assessment can be found elsewhere.[27] Comorbidity in the TS sample was of 42% for OCD and 49% for ADHD.

The replication cohort was composed of 37 nuclear families and three unrelated TS cases, all European, totaling 44 cases (74% males; mean age 20 ± 13 y, and mean age at onset 6 ± 3 y), and 73 unaffected family members as controls (44% males; mean age 42 ± 15 y). Families included eight complete trios, 10 complete nuclear families with sibship data, and 19 incomplete nuclear families. Patients and relatives were interviewed by specialists in TS from the Movement Disorder and Pediatric outpatient clinics at the Hospital Virgen del Rocío in Seville (Spain). Diagnosis and clinical assessment was the same as for the discovery cohort.

The study was approved by all of the Human Subjects Investigation Committees and was conducted according to the principles expressed in the Declaration of Helsinki. All participating adult subjects or the parents of minor subjects (with the assent of the subject) signed an informed consent form before blood collection.

Selection of SNPs and Genotyping

We selected SNPs in the candidate genes and flanking regions 3 kb upstream and downstream. The criteria for selection of SNPs were based on genetic coverage and linkage disequilibrium (LD) patterns from the white population (CEU: Utah residents with ancestry from northern and western Europe) of the HapMap database (HapMap Genome browser release 27; http://hapmap.ncbi.nlm.nih.gov). Tag SNPs were selected with the following criteria: $r^2 > 0.8$ from any other SNP and a minor allele frequency (MAF) greater than 20%. We used the Haploview software (version 4.2) to calculate LD and r^2 between SNPs. In those genes with no tag-SNPs, we used coding-SNPs for the association analysis.

Finally, a total of 506 SNPs (489 tag SNPs and 17 coding-SNPs) were genotyped in the discovery cohort (for details see Table S1 in Supplemental Data). Twenty-five multiplexed assays were designed, using MassARRAY Assay design software (version 3.1). Multiplex SNP genotyping was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, using iPLEX Gold technology from Sequenom (Sequenom Inc., San Diego, CA, USA), according to the manufacturer's protocol. Acquisition of spectra was performed using Sequenom's mass spectrometer (Sequenom Inc.). To analyze the concordance of the genotyping, we duplicated 1% of the samples.

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The SNPs selected for replication were genotyped with TaqMan SNP Genotyping Assay (Applied Biosciences Hispania, Alcobendas, Madrid, Spain) performed in a LightCycler480 instrument (Roche Applied Science, Indianapolis, IN, USA).

Quality Control

Each run of 25 multiplexed assays was carefully inspected with TyperAnalyzer software (Sequenom Inc.), and evident errors, according to the manufacturer's guidelines, were manually corrected to ensure optimal genotyping. Before the association analysis, quality control was applied with PLINK.[28] The SNPs with $P < 1 \times 10^{-4}$ in a Hardy-Weinberg equilibrium test, genotype call rate less than 90%, concordance rate less than 90%, or MAF less than 1% were excluded. Samples with genotyping rate less than 90% and families and SNPs with Mendelian error rates greater than 10% also were excluded.

Genetic Association Analysis

Genotyping data were coded in linkage format using the Matlab application with graphical interface "Sequenom2pedfile" developed by the first author and freely available on Matlab Central (http://www.mathworks.com/matlabcentral/fileexchange/41569-sequenom2pedfile). Family-based association analyses were performed by using the DFAM test available in PLINK, which allows one to analyze jointly affected offspring trios, complete and incomplete nuclear families with sibship data, and unrelated individuals.[28] We also conducted the TDT test to have an estimate of the risk (odds ratio) associated with the transmission of alleles to offspring. The significance threshold for the discovery study was set by dividing 0.05 by the effective number of independent tests (M_{eff}) according to "Genetic Type I Error Calculator" (GEC) (http://statgenpro.psychiatry.hku.hk/gec/). According to the final number of trios available and the significance threshold, and assuming MAF greater than 20%, we estimated with Quanto software the effect size we were powered to detect at 80% (two-sided). Yet, this calculation is approximate and may underestimate the real power, because our sample includes siblings and unrelated individuals as well.

To select the target SNP(s) to be genotyped in the replication cohort, we performed secondary analyses in the discovery cohort, using imputation and functional annotation in an attempt to fine-map causal variants. Gene(s) containing significant SNPs from the association analysis were entirely imputed with IMPUTEv2, using high-quality genotyped SNPs (call rate, > 95%) and the National Center for Biotechnology Information build b37 reference panel "Phase I integrated variant set release (v3)" of the 1,000 Genomes Project.[28,29] Imputed variants with poor quality of imputation (info metric < 0.6) or MAF less than 5% were excluded. The SNPs with a possible regulatory function in the gene(s) of interest were identified by using RegulomeDB (http://www.regulomedb.org).[30] In this study, we considered functional those SNPs that scored category 1 (likely to affect binding and linked to expression of a gene target) or 2 (likely to affect binding). The LD between the significant SNPs and potentially functional SNPs (those with genotypes available) was examined with Haploview. Significant SNPs and linked functional SNPs ($r^2 > 0.8$) were assessed in the replication cohort.

Finally, and as a complementary analysis, we performed an allelic association analysis through chi-square in a case series design to test whether any of the significant SNPs conferred increased risk for OCD and ADHD comorbidities.

Results

After the application of quality control criteria, a total of 442 individuals and 358 SNPs remained for analysis in the discovery cohort. The GEC indicated that the effective number of independent tests was 267, so the significance threshold was set to 1.9×10^{-4}. We estimated that the study was powered to detect alleles with effect sizes that approximated an odds ratio (OR) of 2.3 or greater.

The results for the top-ranked SNPs ($P < 0.05$ nominal) in the DFAM test in the discovery cohort are summarized in Table S2 in Supplemental Data. We found a significant association at rs3096140 in the *GDNF* gene ($P = 1.5 \times 10^{-4}$). The DFAM test indicated that the minor allele "C" (MAF = 31%) was more present in affected individuals than expected under the null hypothesis. The TDT test showed that this allele was significantly overtransmitted to affected offspring (OR = 2.4; $P = 1.7 \times 10^{-4}$). Genotypes of this SNP were confirmed by using a second platform (LightCycler480), and the final genotyping rate was 98%. Additional allelic association analyses in TS cases showed that rs3096140 was not associated with an increased risk for ADHD comorbidity (OR = 0.78; $P = 0.34$). We observed a mild, although not significant, increased prevalence of OCD comorbidity in the minor allele carriers (OR = 1.47; $P = 0.08$).

GDNF was therefore selected as a gene of interest for imputation. From the initial 11 tag-SNPs across this gene, nine remained after quality control (mean call rate = 96%) and therefore were used for imputation. We imputed chromosome 5 from 37 812 779 bp to 37 839 782 bp (GRCh37.p13 assembly), and genotypes for 52 markers, including genotyped and imputed, were available after post-imputation filters. The mean concordance rate of genotyped SNPs was above 94%. RegulomeDB indicated that rs3096140 had minimal binding evidence (category 5) but identified eight SNPs in *GDNF* of categories 1 or 2 (rs11111, rs11749411, rs144626956, rs45455796, rs75135758, rs76568852, rs75310213, rs114812377). However, from these eight only rs11111 was among the 52 markers, and Haploview analysis showed that it was not in LD with our associated SNP ($r^2 = 0$). A table with the post-imputation metrics has been included in the Supplemental Data (Table S3).

Rs3096140 was therefore selected as a target SNP for replication. This SNP was also associated with TS in the replication cohort based on the DFAM test ($P = 0.01$), and the minor allele was also overtransmitted to the affected offspring (OR = 2.7; $P = 0.01$).

Family trees with rs3096140 genotypes for discovery and replication cohorts were depicted with CraneFoot 3.2.1 software (Supplemental Data Figures S1–S6).

Discussion

In the present study, we tested for association 28 candidate genes in a family-based sample with TS patients. As a result, we found that the minor allele of the intronic SNP rs3096140

in the *GDNF* gene is more present in individuals affected by TS in two independent populations of European ancestry.

GDNF is located in 5p, a region that has been associated in genetic linkage studies with TS[24,25] and ADHD.[31] In addition, chromosome 5 is expected to contribute in great proportion to the heritability of TS.[32] However, a previous study with a large number of families genotyped eight SNPs across *GDNF* and failed to find an association.[33] Although that result is contradictory to our findings, possibly they missed the signal that we have detected because rs3096140 was not genotyped. We have observed in our discovery cohort very low LD indexes of this locus with the surrounding markers.

This signal also could have been missed in the recent GWAS by Scharf and collaborators.[34] This large multicenter study included 1285 TS cases of European ancestry, and no markers achieved a genome-wide threshold of significance. This GWAS was powered to detect effect sizes of OR 2.4 or higher, so rs3096140 may not have been interrogated or masked by other effects such as genetic heterogeneity. Although GWA studies have found in recent years a vast number of common variants implicated in numerous diseases, these studies have traditionally accounted for a small proportion of heritability.[35,36] In particular, common variants (MAF > 5%) are expected to explain approximately 80% of the total heritability of TS,[32] and therefore much of this heritability could be overlooked in the GWAS. Family-based analyses offer methods to control for heterogeneity and population stratification and provide valuable information beyond population-based designs such as the effect of variants on disease status according to the pattern of segregation within pedigrees. In fact, combined approaches are nowadays recommended to ascertain the true genetic contribution to traits.[37]

The SNP rs3096140 is located in the intronic region between exons 2 and 3. RegulomeDB scored rs3096140 category 5 (minimal binding evidence), and Haploview analysis showed no linkage disequilibrium with the functional variant rs11111, and thus the association signal detected by rs3096140 could not be explained by a true association with rs11111. Consequently, the putative function of this signal remains to be clarified. This particular SNP also has been related to increased levels of anxiety in a population of 708 white young adults.[38] Given that OCD and anxiety disorders seem to share genetic background,[39] this genetic marker may predispose subjects to suffer from any of the conditions within this disease spectrum. However, in our case series analysis, we did not observe a higher prevalence of either OCD or ADHD in carriers of the minor allele. A mild trend was seen for OCD, so the specific role of this SNP should be addressed in larger cohorts with validated scales.

The GDNF is a potent trophic agent that promotes survival, differentiation, and phenotype of dopaminergic neurons.[40] It is required for the survival of adult substantia nigra and ventral tegmental area dopaminergic neurons,[22] two brain regions with a predominant role in TS.[41] Therefore, a potential alteration of GDNF production associated with the SNP identified in this study could lead to functional modifications of the nigrostriatal pathway. We have recently shown that, in mice, 95% of the striatal GDNF-expressing cells are PV+ interneurons, and the remaining 5% are Chat+ and somatostatin+ interneurons.[26] Children and adults with TS show a 5% decrease in caudate volume,[16] and postmortem studies have

revealed that these reductions are probably attributable to the loss of PV+ and Chat+ interneurons.[14,15] Another study also has provided indications that striatal interneurons produce GDNF to regulate the levels of Sonic hedgehog (Shh), and Shh released by SN cells controls GDNF production. In animals, genetic alteration of this feedback loop leads to cell death of both PV+ and Chat+ striatal interneurons.[42]

The PV+ interneurons are fast-spiking GABAergic cells controlling the spike timing in medium spiny neurons, thereby regulating striatal output.[43–45] Therefore, a deficiency of PV + interneurons caused by a defect in GDNF production might lead to an excessive firing rate of medium spiny neurons. This hyperactivity was one of the main mechanisms proposed as being responsible for tics and OCD-like symptoms in TS/OCD mouse models,[12,13] and, therefore, it may turn out to be a major mechanism in TS in humans.[46]

Our study also has potential limitations. The sample size is not as large as in recent multicenter studies, which limited the power to detect signals of modest effect sizes (OR ≤ 2.2). Also, the genotyping call rate was not satisfactory for many of the studied SNPs, so we cannot discard the possibility that we missed other important signals.

In conclusion, our results support the involvement of *GDNF* in TS in the European population. This gene therefore might be implicated in the pathogenesis of the disorder. Impaired production of GDNF could compromise the survival of parvalbumin interneurons, which are known to be reduced in TS. This defect may alter the excitatory/inhibitory balance in the corticostriatal circuitry, thus giving rise to the typical symptoms of TS. Validation of this marker from other family cohorts is encouraged. Further research is needed to fine-map the causal variant and to confirm this hypothesis.

Acknowledgments

Funding agencies: This study was supported by New Jersey Center for Tourette Syndrome and Associated Disorders (NJCTS), the National Institute of Mental Health (R01MH092293), the Instituto de Salud Carlos III (PI10/01674, PI13/01461, PI14/01823), the Consejería de Economía, Innovación, Ciencia y Empresa de la Junta de Andalucía (CVI-02526, CTS-7685), the Consejería de Salud y Bienestar Social de la Junta de Andalucía (PI-0741/2010, PI-0437-2012, PI-0471-2013), the Sociedad Andaluza de Neurología, the Fundación Alicia Koplowitz, the Fundación Mutua Madrileña and the Jaques and Gloria Gossweiler Foundation. Ismael Huertas Fernández was supported by the “PFIS” program, Pilar Gómez Garre was supported by the “Miguel Servet” program, and Juan Francisco Martín Rodríguez was supported by the “Sara Borrell” program, all 3 from the Instituto de Salud Carlos III.

We thank Francisco Morón, from the Genomic Service of the Instituto de Biomedicina de Sevilla (IBiS), for his technical support. We also thank Cristina Tejera Parrado for her help in creating the figures. This research has been conducted by using samples from the Andalusian Public Health System Biobank (ISCIII-Red de Biobancos RD12/0036/0017).The authors thank the donors and the Andalusian Public Health System Biobank (ISCIII-Red de Biobancos RD12/0036/0017) for the human specimens used in this study.

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