Psychiatry Research 170 (2009) 199–203

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Effect of candidate gene polymorphisms on the course of attention deficit hyperactivity disorder

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ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 25 November 2008
Accepted 30 December 2008

Keywords:
ADHD
Genetics
Dopamine
Serotonin

ABSTRACT

The main aim of this study was to examine the association between attention-deficit hyperactivity disorder (ADHD)-associated genes and the course of ADHD. Subjects were derived from identically designed case-control family studies of boys and girls with ADHD and a genetic linkage study of families with children with ADHD. Caucasian probands and family members with ADHD and with available genetic data were included in this analysis ($N = 563$). The course of ADHD was compared in subjects with and without putative risk alleles (*DRD4* 7-repeat allele, *DAT1* 10-repeat allele, and *5HTTLPR* long allele). The persistence of ADHD (full or subthreshold diagnosis in the last month) was plotted using Kaplan-Meier survival functions and tested with Cox proportional hazard models. Survival analyses revealed that by 25 years of age 76% of subjects with a *DRD4* 7-repeat allele were estimated to have significantly more persistent ADHD compared with 66% of subjects without the risk allele. In contrast, there were no significant associations between the course of ADHD and the *DAT1* 10-repeat allele ($P = 0.94$) and *5HTTLPR* long allele. Our findings suggest that the *DRD4* 7-repeat allele is associated with a more persistent course of ADHD.

1. Introduction

Behavioral and molecular genetic studies indicate that attention-deficit hyperactivity disorder (ADHD) is a complex phenotype influenced by multiple genes of small effect. Multiple candidate gene studies of ADHD have produced substantial evidence implicating several genes in the etiology of the disorder (Faraone et al., 2005; Li et al., 2006; Purper-Ouakil et al., 2005; Yang et al., 2007). For the eight genes for which the same variant has been studied in three or more case– control or family-based studies, seven have shown statistically significant evidence of association with ADHD on the basis of the pooled odds ratio across studies (*DRD4*, *DRD5*, *DAT1 [SLC6A3]*, *DBH*, *HTT [SLC6A4]*, *HTR1B*, and *SNAP25)* (Faraone et al., 2005; Mick and Faraone, 2008). Maher et al.'s (2002) meta-analysis of dopamine system genes showed positive associations of ADHD with *DRD4* and *DRD5*, while *DAT1* did not reach significance ($P = 0.06$). However, the functional implications of these genes remain unclear.

One possibility of the putative functional effect of ADHD-associated genes could be on their impact on the course of ADHD. Prior work suggests that persistent ADHD may have a higher familial loading than remitting forms of the disorder (Biederman et al., 1995; Manshadi et al., 1983): the risk of ADHD among children of ADHD adults was much higher than the risk for ADHD among relatives of children with ADHD (Biederman et al., 1995). This high familial loading in persistent cases of ADHD suggests that genes may play a role in persistent ADHD.

Two studies have examined the effects of genes on functional outcomes into adulthood and have produced inconsistent results. Mill and colleagues (2006) showed longitudinal evidence that risk variants in *DRD4* (7-repeat allele in exon 3 VNTR) and *DAT1* 3′-untranslated region 40 base-pair VNTR (10/10 genotype) predicted poor adult outcomes such as a criminal conviction, evidence of aggression, or long-term unemployment. Barkley et al. (2006b) found that the *DAT1* 9/10 genotype was associated with greater symptoms of ADHD, externalizing scores, and family, educational, and occupational deficits into adulthood. Additionally, the two studies that have examined the effects of genes on the persistence of an ADHD diagnosis have also produced inconsistent results. Shaw and colleagues (2007) found that subjects with at least one copy of the *DRD4* 7-repeat allele were significantly less likely to retain the diagnosis of combined-type ADHD after 6 years. Langley and colleagues (2009) found that the *DRD4* 7-repeat allele was associated with persistent ADHD. Most recently, Franke et al. (2008) found that a 9-6 *DAT1* haplotype was associated with adult ADHD, and Johansson et al. (2007) showed an association between adult ADHD and *DRD5* but

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doi:10.1016/j.psychres.2008.12.016

not *DAT1* or *DRD4*. The inconsistencies among these findings call for additional studies on the molecular genetics of persistence of ADHD.

The main aim of this study was to examine the association between ADHD-associated genes and the course of the disorder. To this end we examined data from large samples of well-characterized youth with ADHD and their affected first-degree relatives who had been genotyped at three loci in three genes implicated in the risk for ADHD: *DRD4*, *DAT1*, and *HTT*. Based on the evidence from longitudinal twin studies that show a strong genetic influence on the stability of ADHD symptoms (Kuntsi et al., 2005; Larsson et al., 2004; Price et al., 2005), we hypothesized that variants in these genes would predict a more persistent course of ADHD.

2. Methods

2.1. Subjects

Subjects were derived from identically designed case-control family studies of boys (Biederman et al., 2006b) and girls (Biederman et al., 2006a) diagnosed with ADHD as well as a genetic linkage study of families with children with ADHD (Faraone et al., 2007). Boys from the family study were reassessed at 4-year and 10-year follow-ups and girls from the family study were reassessed at a 5-year follow-up, while subjects from the genetic linkage study and parents from the family studies were assessed only once. All subjects were Caucasian. Subjects who met full criteria for ADHD at their first (or only) assessment, had a current diagnosis of ADHD at their last (or only) assessment, and who had genetic data were included in this analysis ($N=563$ total; $N=74$ from boys study, $N=128$ from girls study, and $N=361$ from linkage study). Of the 563 subjects, 59% ($N=332$) were male. The mean age of the sample was 19.5 years (standard deviation = 14.4) at subjects' last assessment. Offspring of families made up 77% of the sample ($N=432$), while 23% ($N=131$) were parents. Parents and adult offspring provided written informed consent to participate, and parents also provided consent for offspring under the age of 18. Children and adolescents provided written assent to participate. The human research committee at Massachusetts General Hospital approved this study.

2.2. Assessment procedures

All subjects were assessed with the same assessment battery. Detailed study methodologies for each study had been previously reported (Biederman et al., 2006b; Faraone et al., 2007). Briefly, in all studies, psychiatric assessments relied on the Schedule for Affective Disorders and Schizophrenia for School-Age Children, Epidemiologic (K-SADS-E) (Orvaschel and Puig-Antich, 1987) for subjects younger than 18 years of age and the Structured Clinical Interview for DSM-IV (SCID)(First et al., 1997) (supplemented with modules from the K-SADS-E to assess childhood diagnoses) for subjects 18 years of age and older. Diagnoses were based on independent interviews with the mothers and direct interviews with subjects, except that children younger than twelve years of age were not directly interviewed. We considered a diagnostic criterion positive if it was endorsed in either interview.

The interviewers were blind to the subject's ascertainment source and any prior assessments. The interviewers had undergraduate degrees in psychology and were extensively trained. First, they underwent several weeks of classroom style training, learning interview mechanics, diagnostic criteria and coding algorithms. Then, they observed interviews by experienced raters and clinicians. They subsequently conducted at least six practice (non-study) interviews and at least three study interviews while being observed by senior interviewers. Trainees were not permitted to conduct interviews independently until they executed at least three interviews that achieved perfect diagnostic agreement with an observing senior interviewer. We computed kappa coefficients of agreement by having experienced, board certified child and adult psychiatrists and licensed clinical psychologists diagnose subjects from audio taped interviews. Based on 500 assessments from interviews of children and adults, the kappa coefficient for ADHD was 0.88.

We considered a diagnosis of ADHD present if DSM diagnostic criteria were unequivocally met (DSM-III-R for boys study baseline and 4-year follow-up and girls study baseline; DSM-IV for boys study 10-year follow-up, girls study 5-year follow-up, and the genetic linkage study). A committee of board-certified child and adult psychiatrists who were blind to the subject's ADHD status, referral source, and all other data resolved diagnostic uncertainties. We estimated the reliability of the diagnostic review process by computing kappa coefficients of agreement for clinician reviewers. For ADHD, the reliability between individual clinicians and the review committee was a kappa of 1.0.

2.3. Genotyping

Genotyping of the *HTT* polymorphism and *DRD4* VNTRs was performed using the following protocol. Genomic DNA (5 ng) was amplified in a 7 μl reaction using KlenTaq DNA Polymerase (0.2 U; DNA Polymerase Technology, Inc., St. Louis, Missouri, USA), the proprietary KlenTaq Buffer (1X), dNTPs (200 μM each), glycerol (5% for *HTT* and 10% for DRD4), Betaine (1 M) and the marker specific primers (0.2 μM). The *DRD4* VNTR primers were as follows: DRD4-EX03B-F VIC-GACCGCGACTACGTGGTCTACTC, DRD4-EX03B-R CTCAGGACAGGAACCCACCGAC. The DRD4-EX03B-R primer also contains a proprietary tail that helps stabilize the amplified product. The *HTT* promoter VNTR xprimers were as follows: SLC6A4_PRO-F 6FAM-ATGCCAGCACCTAACCCCTAATGT, SLC6A4_PRO-R GGACCGCAAGG-TGGGCGGGA. Amplification was performed with the following protocol: thirteen cycles of denaturation for 30 s at 93 °C, annealing for 30 s beginning at 61.5 °C for the *HTT* marker and 69.5 °C for the *DRD4* marker and dropped 0.5 ° C every cycle and primer extension at 72 °C for 30 s; 37 cycles of denaturation for 30 s at 93 °C, annealing for 30 s at 55 ° C for the *HTT* marker and 63 °C for the *DRD4* marker and primer extension at 72 °C for 30 s; 72 °C for 1 h.

Genotyping of *DAT1* VNTRs used the following protocol. Genomic DNA (5 ng) was amplified in a of 7 μl reaction using HotStarTaq DNA Polymerase (0.2 U; Qiagen, Valencia, California, USA), the proprietary HotStarTaq Buffer (1X), dNTPs (200 μM each), and the marker specific primers (0.2 μM). Primers were as follows: DAT1-F 6FAM–TGTGGTGTAGGGA ACGGCCTGAG, DAT1-R CCTCCTGGAGGTCACGGCTCAAGG. The DAT1-R primers also contain the proprietary tail. Amplification for *DAT1* was performed as follows. Samples were heated at 92 ° C for 9 min to activate the HotStarTaq Polymerase. This is followed by twelve cycles of denaturation for 30 s at 93 °C, annealing for 30 s beginning at 64.5 °C and dropped 0.5 ° C every cycle and primer extension at 72 °C for 30 s; 37 cycles of denaturation for 30 s at 93 °C, annealing for 30 s at 58 °C and primer extension at 72 °C for 30 s; 72 °C for 1 h.

Amplified products were pooled and combined with size standard (LIZ-500, Applied Biosystems, Foster City, California, USA) before being analyzed on an ABI-3730 (Applied Biosystems). GeneMapper v3.5 (Applied Biosystems) was used to analyze the raw results from the ABI3730, however, a genotype was not considered final until two laboratory personnel had independently checked (and corrected) the GeneMapper results and both individuals were in agreement.

Additional quality control parameters were considered. Rates of missing alleles fell within the acceptable range (less than 3% for all genes). Hardy-Weinberg equilibrium was also acceptable (all $P>0.10$), and allele frequencies were consistent with the literature.

2.4. Statistical analysis

The course of ADHD was compared in subjects with and without putative risk alleles (*DRD4* 7-repeat allele, *DAT1* 10-repeat allele, and *HTT* long allele). In keeping with previous studies (Barkley et al., 2006b; Langley et al., 2009; Mick and Faraone, 2008; Mill et al., 2006; Shaw et al., 2007), the risk genotypes were defined as at least one copy of the *DRD4* 7-repeat allele, at least one copy of the *HTT* long allele, and two copies of the *DAT1* 10-repeat allele. Persistence of ADHD was defined as subjects meeting full or subthreshold (more than half of the symptoms required for a full diagnosis) DSM criteria for ADHD in the month prior to the subject's assessment (most recent assessment if more than one). That is, while subjects with only one assessment met criteria for a lifetime diagnosis of ADHD, they were considered to be in remission if they did not meet full or subthreshold DSM criteria during the month prior to their assessment. Our definition of remission is more accurately described as "symptomatic remission" (Biederman et al., 2000; Keck et al., 1998) and is consistent with numerous prospective follow-up studies of ADHD (Faraone et al., 2006). Analyses were truncated at age 25 years because of the very few failures (i.e., ADHD remission) that occurred after that age. That is, subjects who had an offset of ADHD after age 25 were considered persistent. A DSM-IV ADHD diagnosis was available to define persistence for the large majority of subjects at their last assessment ($N=523$); otherwise, DSM-III-R criteria were used ($N=40$). Biederman and colleagues (1997) showed that 93% of children with a DSM-III-R diagnosis also received a DSM-IV diagnosis.

The persistence of ADHD was plotted using Kaplan-Meier survival functions and tested with Cox proportional hazard models. On the plots, vertical drops indicate the proportion of the sample whose ADHD had remitted at a particular age. This survival analysis censors subjects based on their age at last interview, which is essential given the large range of ages of this sample. The failure event of the survival functions was remission of ADHD (i.e., non-persistence) and the analysis-time variable was the age of offset of ADHD (the oldest age reported if the subject had multiple assessments). We controlled for study of ascertainment in the Cox regression models. To account for the non-independence of family members, we used the Huber (Huber, 1967) correction to produce robust variances. All tests were two-tailed with alpha set at 0.05. Statistical analyses were conducted using STATA (Stata Corporation, 2005).

3. Results

Table 1 shows demographic characteristics and comorbid disorders of the sample. Of the 563 subjects, 13 were missing *DRD4* data, 11 were missing *DAT1* data, and 7 were missing *HTT* data. Rates of risk genotypes are presented here as a comparison to other studies of subjects with ADHD or population studies. Table 2 shows the distribution of genotypes for *DRD4*, *DAT1*, and *HTT*. Thirty-one percent (171/550) of subjects had at least one copy of the *DRD4* 7-repeat allele, 55% (301/552) had two copies of the *DAT1* 10-repeat allele, and 79% (442/556) at least one copy of the *HTT* long allele. Neither age at interview nor sex was associated with any of the risk alleles (all $P>0.05$). Genotypes were in Hardy-Weinberg equilibrium (all $P>0.10$).