Evaluation of single nucleotide polymorphisms in the miR-183-96-182 cluster in adulthood attention-deficit and hyperactivity disorder (ADHD) and substance use disorders (SUDs)

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Received 24 January 2013; received in revised form 28 May 2013; accepted 5 July 2013

KEYWORDS
ADHD; SUD; miRNA; Case-control association study; SNP; HTR1B

Abstract
Attention deficit-hyperactivity disorder (ADHD) is a neuropsychiatric disorder characterized by inappropriate and impaired levels of hyperactivity, impulsivity and inattention. Around 75% of adults with ADHD show comorbidity with other psychiatric disorders such as disruptive behavior disorders or substance use disorders (SUDs). Recently, there has been growing interest in studying the role of microRNAs (miRNAs) in the susceptibility to complex disorders. Interestingly, converging evidence suggests that single nucleotide polymorphisms (SNPs) within miRNAs or miRNA target sites may modulate the miRNA-mediated regulation of gene expression through the
1. Introduction

Attention deficit-hyperactivity disorder (ADHD) is a neuropsychiatric disorder affecting 4-8% of children characterized by inappropriate and impaired levels of hyperactivity, impulsive behavior, and inattention, with an estimated heritability of 76% (Faraone and Mick, 2010; Faraone et al., 2005; Polanczyk et al., 2007). In approximately 65% of ADHD cases, the disorder persists into adulthood either as a full condition or in partial remission (Faraone et al., 2006; Lara et al., 2009). Around 75% of adults with ADHD show comorbidity with other psychiatric disorders such as anxiety, affective disturbances, or substance use disorders (Fayad et al., 2007; McGough et al., 2005). Specifically, individuals whose ADHD persists into adulthood have a 50% lifetime risk of developing any SUD and patients with SUD present a higher prevalence of ADHD than the general population. In a recent meta-analysis, the mean ADHD prevalence in SUD patients was estimated to be 25.3% in adolescents and 21.0% in adults (Biederman et al., 1995; Daigre et al., 2013a, 2013b; DeMilio, 1989; van Emmerik-van Oortmerssen et al., 2012; Wilens and Biederman, 2006). This link between ADHD and SUDs has been documented in family members of both adults and children with ADHD. In this regard, follow-up studies have shown a higher risk for SUD in adults with ADHD or in adults that had ADHD as children than in individuals without ADHD (Biederman et al., 2012; Kessler et al., 2006; Klein et al., 2012; Mannuzza et al., 1991). In addition, elevated rates of SUD have been described as relatives of children with ADHD, while the offspring of parents with SUD are at increased risk for ADHD or related symptoms (Biederman et al., 1992, 2008; Wilens et al., 1995). Despite the high incidence of SUD in subjects with ADHD and the studies that consistently show a genetic basis for the co-occurrence of these two conditions (Groman et al., 2009), little is known about the specific genetic factors that are involved in the joint development of these disorders. Until today, research has mainly focused on candidate genes of the dopamine, serotonin or norepinephrin neurotransmitter systems, showing controversial results (Arnsen and Pliszka, 2011; Banaschewski et al., 2010; Wu et al., 2012). Recently, Carpentier et al. (2013) evaluated the association of six candidate genes with ADHD and/or SUDs and identified both shared as well as ADHD-specific vulnerability factors.

Besides the genetic background, co-occurrence of other major psychiatric disorders, such as conduct disorder (CD) or oppositional defiant disorder (ODD), are also risk factors for the development of SUD in ADHD patients. Along this line, among the patients with ADHD that are more vulnerable to develop SUD later in life are those who have comorbid CD or ODD. Additionally, there is evidence that co-occurrence of ADHD and CD symptoms contributes in an epistatic manner to a more severe form of SUD as compared to ADHD or CD alone (Flory et al., 2003; Katusic et al., 2005; Klein et al., 2012; Kollins, 2008).

More recently, there has been growing interest in studying the role of post-transcriptional regulatory elements in the susceptibility to different complex disorders. MicroRNAs (miRNAs) are 18-25 nucleotides single-stranded non-coding RNAs that play an important role in the regulation of gene expression at the post-transcriptional level (Lewis et al., 2003). Mature miRNAs bind to target mRNAs through a 3′UTR interaction region of about 6-8 nucleotides. Once the interaction is established, and depending on the degree of complementarity between the two strands, mRNA translation is repressed either by interference with ribosome binding or by destabilization or degradation of the mRNA molecule (Prosser et al., 2011; Rana, 2007). Through this mechanism, miRNAs regulate approximately 60% of human protein-coding gene expression at the posttranscriptional level (Ambros, 2004; Friedman et al., 2009; Lewis et al., 2005). Computational estimations indicate that there are more than 800 miRNAs in the human genome (Griffiths-Jones et al., 2008) and the study of their expression profiles has contributed to the understanding of the role of these non-coding RNA molecules in different biological processes found altered in complex diseases, such as cell development, differentiation or oncogenesis (Barbarotto et al., 2008). In the central nervous system, miRNAs are abundant and participate in neuronal survival, development, differentiation and neuronal function (Schatt et al., 2006; Vo et al., 2005). Also, several studies on their possible involvement in different psychiatric disorders, such as schizophrenia, bipolar disorder or autism have shown promising results (Abelson et al., 2005; Beveridge et al., 2008, 2010; Fiore et al., 2008; Hunsberger et al., 2009; Jensen et al., 2009; Shi et al., 2012; Wu et al., 2010). In this context, alterations in miRNA profiles have been described in postmortem brain tissues from schizophrenia patients (Beveridge et al., 2008, 2010; Gardiner et al., 2012; Kim et al., 2010). Along this line, Lai et al. (2011) evaluated the expression profile of 365 miRNAs in leukocytes and identified seven miRNAs with differential expression between schizophrenia patients and controls, alterations that were subsequently replicated in an independent sample for hsa-miR-34. Note-worthy, aberrant expression profiles of miRNAs were also altered of the miRNA maturation, structure or expression pattern as well as the silencing mechanisms of target genes. Genetic studies and animal models support the involvement of the serotonin receptor (HTR1B) in ADHD. We evaluated the contribution of one SNP in the miR-96 target site at HTR1B and eight tagSNPs within the genomic region containing this miRNA in 695 adults with ADHD (266 and 396 subjects with and without comorbid SUD, respectively), 403 subjects with SUD without life-time diagnosis of ADHD and 485 sex-matched controls from Spain. Single and multiple marker analyses revealed association between two SNPs located at the 3′ region of miR-96 (rs2402959 and rs6965643) and ADHD without SUD. Our results provide preliminary evidence for the contribution of two sequence variants at the mir-183-96-182 cluster to ADHD without comorbid SUD, and emphasize the need to take comorbidities into account in genetic studies to minimize the effect of heterogeneity and to clarify these complex phenotypes.
described in autism spectrum disorders (hsa-miR-23a, hsa-miR-134 and hsa-miR-146b) and Tourette's syndrome (hsa-miR-189) (Abelson et al., 2005; Talebizadeh et al., 2008).

Interestingly, converging evidence suggests that single nucleotide polymorphisms (SNPs) within miRNAs or miRNA target sites may modulate the miRNA-mediated regulation of gene expression (Borel and Antonarakis, 2008; Saunders et al., 2007). The first demonstration that mutations in a miRNA target site may have phenotypic consequences came from a study of Tourette's syndrome, where a SNP within the binding site for miR-189 at the 3' UTR of SLTRK1 facilitates the miRNA-mediated downregulation of the gene (Abelson et al., 2005).

Thus, genetic changes in post-transcriptional regulatory elements might lead to variation in the dosage of proteins and contribute to the susceptibility of different disorders, as described for papillary thyroid carcinoma, Parkinson's disease, asthma, and anxiety disorders or schizophrenia (Gong et al., 2013; Jazdzewski et al., 2008; Muinos-Gimeno et al., 2009; Tan et al., 2007; Wang et al., 2008). Although no previous studies have analyzed SNPs within miRNAs in ADHD, it is worth mentioning that Kovacs-Nagy et al. evaluated in silico the effect in miRNA binding of two SNPs located in the putative miRNA-binding site of the SNAP-25 gene and found that both sequence variants, rs3746544 and rs1051312, previously associated with ADHD, may influence hsa-miR-510 and hsa-miR-641 binding and, thus, have an effect on SNAP-25 expression (Kovacs-Nagy et al., 2011).

In addition, recent studies suggest that a sequence variant in the 3' UTR of the serotonin receptor 1B gene (HTR1B; rs13212041), which disrupts the binding site for miR-96 and, thus, moderates the miR-96-mediated regulation of its expression, is associated with aggressive behaviors that often co-occur with ADHD (Conner et al., 2010; Jensen et al., 2009). Interestingly, genetic studies and animal models support the involvement of HTR1B in ADHD and in the methylphenidate-induced locomotor activity in rat (Banerjee et al., 2012; Brunner et al., 1999; Guimaraes et al., 2009; Hawi et al., 2002; Quist et al., 2003; Saudou et al., 1994). Based on the hypothesis that sequence variants within this miRNA or its target site in HTR1B may contribute to the genetic susceptibility to ADHD as well as to comorbid CD, ODD and/or SUD, we genotyped nine sequence variants within the genomic regions containing miR-96 or its target site in HTR1B in 695 adults with ADHD and 485 sex-matched unrelated controls from Spain. Afterwards, those markers found associated with ADHD were further evaluated in an independent sample of SUD without life-time diagnosis of ADHD.

2. Experimental procedures

2.1. Patients and controls

2.1.1. ADHD sample

The ADHD sample consisted of 695 adult ADHD patients (mean age 37.17 ± 10.65 years and 68.78% males (n=478)) recruited and evaluated at the Psychiatry Department of the Hospital Universitari Vall d'Hebron (Barcelona, Spain) according to DSM-IV TR criteria. ADHD diagnosis was based on the Spanish version of the Conners Adult ADHD Diagnostic Interview for DSM-IV (CAADID) (Ramos-Quiroga et al., 2012). Thirty-seven percent of ADHD patients (n=261) fulfilled diagnosis criteria for SUD and 21% for disruptive behavior disorders (CD and/or ODD; n=144). Comorbidity was assessed by Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and SCID-II) (First et al., 1999a, 1999b). Oppositional Defiant Disorder (ODD) during childhood and adolescence was retrospectively evaluated with the Schedule for Affective Disorders and Schizophrenia for School-Age Children (Kiddie SADS) present and lifetime version (K-SADS) (Orvaschel et al., 1995). The level of impairment was measured by the Clinical Global Impression (CGI) included in the CAADID Part II and the Sheeher Disability Inventory. Exclusion criteria for the ADHD patients cohorts were IQ ≤ 80; pervasive developmental disorders; schizophrenia or other psychotic disorders; the presence of mood, anxiety or personality disorders that might explain ADHD symptoms; birth weight ≤ 1.5 kg; and other neurological or systemic disorders that might explain ADHD symptoms.

2.1.2. SUD sample

The SUD sample consisted of 403 adults (mean age 39.9 ± 9.20 years and 73.95% males (n=298)) recruited and evaluated at the Drug Unit of the Psychiatry Department at the Hospital Universitari Vall d’Hebron (Barcelona, Spain) with the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (First et al., 1999b). All patients fulfilled DSM-IV criteria for drug dependence. None of them met lifetime diagnostic criteria for ADHD according to the Spanish version of the Conners Adult ADHD Diagnostic Interview for DSM-IV (CAADID) (Ramos-Quiroga et al., 2012).

2.1.3. Controls

The control sample (mean age 52.61 ± 14.80 years and 74.23% males (n=360)) consisted of blood donors matched for sex with the ADHD group in which DSM-IV lifetime symptomatology was excluded under the following criteria: (1) not having been diagnosed with ADHD and (2) answering negatively to the lifetime presence of the following DSM-IV ADHD symptoms: (a) often has trouble keeping attention on tasks, (b) often loses things needed for tasks, (c) often fidgets with hands or feet or squirms in seat and (d) often gets up from seat when remaining in seat is expected. None of them had injected drugs intravenously.

All patients and controls were Spanish and Caucasian (Table 1). The study was approved by the ethics committee of our institution and informed consent was obtained from all subjects in accordance with the Helsinki Declaration.

2.2. DNA isolation, selection of SNPs and genotyping

Genomic DNA was isolated either from saliva using the Oragene-DNA self-collection kit from DNA Genotek (DNA Genotek Inc., Ottawa, Canada) or from peripheral blood lymphocytes by the salting-out procedure (Miller et al., 1988). DNA concentrations were determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon).

Since miR-96 is clustered in an intronic region with two other miRNA genes, miR-183 and miR-182, and of all of them are transcribed as a single polycistronic transcript, for the tagSNP selection we considered a genomic region including the miR-183-96-182 cluster plus 10 kb proximal and 5 kb distal flanking sequences (chr7:129192459–129212090). SNP selection was based on genetic coverage criteria (r² > 0.80) considering SNPs with a minor allele frequency (MAF) > 0.05 from the CEPH panel of the HapMap database (www.hapmap.org; release 28) and using the Haploview v.4.2 software (Barrett et al., 2005). Under these conditions, we selected a total of six tagSNPs covering, in terms of linkage disequilibrium, the genomic region that contains the miR-183-96-182 cluster (Table S1; Figure 1). Three additional SNPs located within the stem loop of the miR-96 precursor (pre-miR-96; rs73159662 and rs41274239) or at the miR-96 binding site of the HTR1B gene (rs13212041) were also included in the analysis (Figure 1). Genotyping was performed using the Sequenom technol
ogy at the Barcelona node of the Spanish National Genotyping Center (CeGen; www.cegen.org). Two CEPH DNA samples (NA11992 and NA11993) were included in the different genotyping assays, and a concordance rate of 100% with HapMap data was obtained. Replicates were also included in all plates as an additional quality control, and genotype concordance was also 100%.

2.3. Statistical analysis

To better understand the genetic contribution to adulthood ADHD we first analyzed the whole ADHD sample and afterwards considered comorbid disruptive behavior disorders or SUD in the association study. Those markers found associated with or without drug dependence in the ADHD sample were subsequently evaluated in an independent sample of SUD without lifetime diagnosis of ADHD. The analysis of minimal statistical power was performed post hoc using the Genetic Power Calculator software (Purcell et al., 2003) assuming an odds ratio (OR) of 1.3, prevalence of 0.05, significance level of 0.05 and the lowest MAF of 0.112. Genetic stratification in our sample was previously discarded by analyzing a set of 48 anonymous SNPs with three different approaches that include the STRUCTURE software, the FST coefficient and the Prichard and Rosenberg method (Fernandez-Castillo et al., 2013; Ribases et al., 2009, 2008; Rosenberg et al., 2005). The analysis of Hardy-Weinberg equilibrium and the comparison of genotype frequencies under a log-additive model of inheritance were performed using the SNP association analysis software (SNPassoc R package, Gonzalez et al., 2007). Only SNPs following a genotypic distribution in the Hardy-Weinberg equilibrium (p > 0.05) in the control sample were included in the analysis. To minimize multiple testing and type I errors, we decide a priori to restrict the multiple-marker analysis to those SNPs displaying

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Descriptive characteristics of the Caucasian sample of ADHD, SUD and controls.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADHD sample, N=695</td>
</tr>
<tr>
<td><strong>Gender N (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>478 (68.78)</td>
</tr>
<tr>
<td>Female</td>
<td>217 (31.22)</td>
</tr>
<tr>
<td><strong>Age (mean and SD)</strong></td>
<td></td>
</tr>
<tr>
<td>37.17 ± 10.65</td>
<td>37.61 ± 8.94</td>
</tr>
<tr>
<td><strong>ADHD subtypes N (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Combined type</td>
<td>425 (61.5)</td>
</tr>
<tr>
<td>Inattentive type</td>
<td>237 (34.10)</td>
</tr>
<tr>
<td>Hyperactive/impulsive type</td>
<td>23 (3.31)</td>
</tr>
<tr>
<td>Sub-threshold</td>
<td>1 (0.14)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (1.29)</td>
</tr>
<tr>
<td><strong>SUD N (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Total SUD</td>
<td>261</td>
</tr>
<tr>
<td>Cocaine</td>
<td>137 (52.5)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>152 (58.2)</td>
</tr>
<tr>
<td>Cannabis</td>
<td>190 (72.5)</td>
</tr>
<tr>
<td>Opiates</td>
<td>12 (4.6)</td>
</tr>
<tr>
<td>Amphetamines and metamphetamines</td>
<td>31 (11.9)</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>17 (6.5)</td>
</tr>
<tr>
<td>Others</td>
<td>41 (15.7)</td>
</tr>
<tr>
<td><strong>Disruptive behavior disorders (CD and/or ODD)</strong></td>
<td></td>
</tr>
<tr>
<td>Non CD and/or ODD</td>
<td>458 (65.9)</td>
</tr>
<tr>
<td>CD and/or ODD</td>
<td>144 (20.72)</td>
</tr>
<tr>
<td>Non evaluated</td>
<td>93 (13.38)</td>
</tr>
</tbody>
</table>

*) Abuse and/or dependence.

Figure 1  Location of the six markers selected in the miR-183-96-182 cluster region plus 10 kb proximal and 5 kb distal sequences (chr7:129192459-129212090).
nominal association in the single-marker study. Haplotypes were estimated and assigned to individuals using the PHASE 2.0 software (Stephens et al., 2001). Subsequently, we compared haplotype frequencies under a log-additive model of inheritance with the SNPassoc R package (Gonzalez et al., 2007). Epistatic effects were evaluated by taking the two genetic markers of interest (SNPs or haplotypes) and comparing two different regression models by a likelihood ratio test with the SPSS 15.0 statistical package (SPSS Inc., Chicago, USA). In the first model, we took the affection status as a dependent variable and the two risk genetic markers as predictive variables. In the second model, we included the interaction between both genetic factors as an independent variable in the logistic regression model. The Bonferroni correction for multiple testing considering seven SNPs corresponds to a significance threshold of \( p < 0.007 \). The SNP Function Prediction utility was used to detect potential functional effects of the associated variants (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm).

3. Results

We performed a case-control association study in a sample of 695 adult ADHD patients and 485 unrelated controls considering eight SNPs located within the genomic region that contains miR-96 (Figure 1) and one SNP in the 3′ UTR miRNA binding site of the HTR1B gene. Two out of these nine SNPs (rs73159662 and rs41274239) were monomorphic in our sample and were excluded from the analysis (Table 51). The minimal statistical power of the sample considering a log-additive model of inheritance was 40.2%.

In the single-marker analysis, the comparison of genotype frequencies between the whole ADHD sample and the control group showed no significant differences (\( p > 0.05 \)). We then considered the presence or absence of comorbid disruptive behavior disorders (CD and/or ODD) or SUD. No significant differences were observed for any SNP when we compared ADHD with or without comorbid disruptive behavior disorders with controls, or when ADHD with SUD was considered (data not shown). However, two SNPs located downstream from miR-96 displayed nominal association with ADHD without comorbid SUD (rs2402959: \( p = 0.047, \text{OR} = 1.25 \) (1.0-1.56) and rs6965643: \( p = 0.029, \text{OR} = 1.31 \) (1.02-1.66); Table 2). Consistently, these differences remained significant for rs6965643 when ADHD with neither SUD nor disruptive behavior disorders was compared with the control group (\( p = 0.038; \text{OR} = 1.31 \) (1.01-1.69)).

The two SNPs within the flanking region of miR-96 identified in the single-marker study were further investigated using a haplotype-based test in the ADHD without SUD dataset. The analysis of multiple-markers under a log-additive model of inheritance revealed overrepresentation of the rs2402959/rs6965643A haplotype (\( p = 0.037; \text{OR} = 1.25 \) (1.01-1.56)) and underrepresentation of the rs2402959/rs6965643G haplotype (\( p = 0.024; \text{OR} = 1.36 \) (1.04-1.81)) in the sample of ADHD without SUD (Table 3a). Consistently, these differences remained significant when the subgroups of ADHD patients with and without SUD were compared to each other (rs2402959/rs6965643A (\( p = 0.02; \text{OR} = 1.32 \) (1.03-1.70)) and rs2402959/rs6965643G (\( p = 0.03; \text{OR} = 1.40 \) (1.02-1.93); data not shown). Under-representation of the rs2402959C/rs6965643G haplotype was also identified in the ADHD group with neither SUD nor disruptive behavior disorders (\( p = 0.01; \text{OR} = 1.44 \) (1.07-1.96); Table 3b).

### Table 2

<table>
<thead>
<tr>
<th>Locus</th>
<th>Marker</th>
<th>Region</th>
<th>Genotypes (11-12-22)</th>
<th>Controls N (%)</th>
<th>Cases N (%)</th>
<th>Genotypes (11-12-22)</th>
<th>Controls N (%)</th>
<th>Cases N (%)</th>
<th>Genotypes (11-12-22)</th>
<th>Controls N (%)</th>
<th>Cases N (%)</th>
<th>Genotypes (11-12-22)</th>
<th>Controls N (%)</th>
<th>Cases N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR96</td>
<td>rs2402959</td>
<td>11</td>
<td>120 (46.1)</td>
<td>131 (49.4)</td>
<td>62 (23.4)</td>
<td>120 (46.1)</td>
<td>131 (49.4)</td>
<td>62 (23.4)</td>
<td>120 (46.1)</td>
<td>131 (49.4)</td>
<td>62 (23.4)</td>
<td>120 (46.1)</td>
<td>131 (49.4)</td>
<td>62 (23.4)</td>
</tr>
<tr>
<td></td>
<td>rs6965643</td>
<td>12</td>
<td>118 (50.9)</td>
<td>130 (53.2)</td>
<td>53 (20.4)</td>
<td>118 (50.9)</td>
<td>130 (53.2)</td>
<td>53 (20.4)</td>
<td>118 (50.9)</td>
<td>130 (53.2)</td>
<td>53 (20.4)</td>
<td>118 (50.9)</td>
<td>130 (53.2)</td>
<td>53 (20.4)</td>
</tr>
<tr>
<td></td>
<td>rs4626538</td>
<td>22</td>
<td>119 (49.6)</td>
<td>132 (52.0)</td>
<td>51 (20.0)</td>
<td>119 (49.6)</td>
<td>132 (52.0)</td>
<td>51 (20.0)</td>
<td>119 (49.6)</td>
<td>132 (52.0)</td>
<td>51 (20.0)</td>
<td>119 (49.6)</td>
<td>132 (52.0)</td>
<td>51 (20.0)</td>
</tr>
</tbody>
</table>

*P-values (OR – 95% CI) are based on a log-additive model of inheritance. The Cochran-Armitage’s Trend Test is significant when OR > 1, inverted score is shown.*
mediated inhibition of and which has a role in the attenuation of the miR-96-aggression-related phenotypes that often accompany ADHD polycistronic transcript, the identification of which occurred on 7q32.2 with two other miRNA genes, miR-183 and miR-182. Since these three miRNAs are transcribed as a single cluster , the identification of miR-96 is clustered in an intergenic region of the HTR1B gene, previously associated with disorders and 485 controls.

### Table 3  
Multiple-marker association study (rs2402959/rs6965643) in (a) a clinical sample of 396 adult ADHD patients without SUD and 488 controls, and (b) a clinical sample of 318 adult ADHD patients without SUD or disruptive behavior disorders and 485 controls.

<table>
<thead>
<tr>
<th>Marker haplotype</th>
<th>Controls N (%)</th>
<th>Cases N (%)</th>
<th>p-value (OR – 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Copies</td>
<td>1 Copy</td>
<td>2 Copies</td>
</tr>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>332 (68.5)</td>
<td>143 (29.5)</td>
<td>10 (2.1)</td>
</tr>
<tr>
<td>TA</td>
<td>48 (9.9)</td>
<td>225 (46.4)</td>
<td>212 (43.7)</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>332 (68.5)</td>
<td>143 (29.5)</td>
<td>10 (2.1)</td>
</tr>
<tr>
<td>TA</td>
<td>48 (9.9)</td>
<td>225 (46.4)</td>
<td>212 (43.7)</td>
</tr>
</tbody>
</table>

*aWhen OR < 1, inverted score is shown.

To confirm that the association between ADHD and miR-96 was dependent on the SUD condition and exclusive of patients without comorbid SUD, we considered an additional sample of 403 SUD patients in which lifetime ADHD was excluded. Consistently, no association between SUD and rs2402959 or rs6965643 was observed neither in the single-marker nor in the multiple-marker analyses (p > 0.05; data not shown).

We then evaluated the possible interaction between the risk haplotype identified at 3’ from miR-96 (rs2402959/rs6965643) and rs13212041 in the microRNA target site of the HTR1B gene, and found no evidence supporting the existence of epistatic effects between these sequence variants in the risk to develop ADHD without comorbid SUD (data not shown).

### 4. Discussion

As far as we know, this is the first case-control association study in ADHD that evaluates the role of SNPs in miRNAs or miRNA target sites and, thus, with a potential involvement in the miRNA-mediated modulation of gene expression. Under the hypothesis that common variations in miR-96 or in its target binding site in the HTR1B gene may be related to the susceptibility to ADHD, we focused our attention on SNPs covering the chromosomal region that contains this miRNA. We also considered a variant located at the 3’UTR region of the HTR1B gene, previously associated with aggression-related phenotypes that often accompany ADHD and which has a role in the attenuation of the miR-96-mediated inhibition of HTR1B expression (Conner et al., 2010; Jensen et al., 2009).

Our results provide preliminary evidence for the contribution of two sequence variants downstream from miR-96 to ADHD without comorbid SUD. However, it is worth to mention that miR-96 is clustered in an intergenic region on 7q32.2 with two other miRNA genes, miR-183 and miR-182. Since these three miRNAs are transcribed as a single polycistronic transcript, the identified sequence variants may affect the expression profiles of the entire miR-183-96-182 cluster. Interestingly, alterations in this miRNA cluster, that is expressed in the central nervous system and shows brain region-specific expression patterns (Juhila et al., 2011; Xu et al., 2007), could contribute to ADHD susceptibility through their effect not only on the post-transcriptional regulation of HTR1B, but also on other validated target genes such as ADCY6, CLOCK and/or DSIP, involved in the regulation of the circadian rhythm, or RARG, that participates in the control of dopamine signaling pathway (Baird et al., 2012; Feng et al., 2005; Goodman, 1998; Krezel et al., 1998; Saus et al., 2010; Xu et al., 2007). All these genes can be considered good candidates for being involved in ADHD.

The association between the miR-183-96-182 cluster and ADHD without comorbid SUD suggests the existence of a distinct genetic load between different ADHD subgroups and argues for specific genetic risks in ADHD patients with and without SUD. Since regular treatment with methylphenidate is less effective in ADHD patients with comorbid SUD (Faraone et al., 2007; Faraone and Upadhyaya, 2007; Huss et al., 2008; Wilens et al., 2003), confirmation of distinct genetic factors that would allow discrimining between individuals with and without increased SUD risk would be particularly relevant from the preventive and therapeutic points of view (Kollins, 2008; Wilens et al., 2003). Because the genetic basis of ADHD remains largely unknown, this finding also points to the assessment of the ADHD comorbidity as an alternative approach to identify distinctive, homogeneous and consistent subgroups of ADHD that could facilitate the identification of underlying genetic factors. Along this line, clinical heterogeneity and differences in the frequency of comorbid disorders co-occurring with ADHD across populations may have contributed to the inconsistent and sometimes divergent results observed in previous association studies.

Although miRNAs are essential for synaptic plasticity, neuronal differentiation, development and maintenance (Barbato et al., 2007; Kim et al., 2007; Numakawa et al., 2011), little is known about their involvement in ADHD. However, recent studies have attempted to evaluate the impact of miRNAs in other psychiatric disorders and have reported alterations in miRNA biogenesis and/or expression in brain and peripheral blood cells in schizophrenia, bipolar disorder, major depression and/or suicide patients (Beveridge et al., 2008, 2010; Gardiner et al., 2012; Kim
et al., 2010; Lai et al., 2011; Rong et al., 2011; Smalheiser et al., 2012; Xu et al., 2010; Zhu et al., 2009). Because nicotine, alcohol or cocaine selectively modulates the expression of multiple miRNAs, the miRNA-mediated gene regulation may also underlie the molecular mechanisms involved in drug dependence (Hollander et al., 2010; Huang and Li, 2009; Im et al., 2010; Li and van der Vaart, 2011; Pietrzykowski et al., 2008). Finally, in addition to expression studies, different research groups have investigated genetic variants within or nearby brain-expressed miRNAs through case-control designs and found significant associations with schizophrenia, major depression or anxiety disorders (Hansen et al., 2007; Muinos-Gimeno et al., 2009; Saus et al., 2010).

However, our results should be interpreted in the context of several limitations. First, the relationship between ADHD and the miR-183-96-182 cluster was only identified when ADHD patients without SUD were considered, but not in ADHD with comorbid SUD or in SUD alone. These results are in line with previous studies pointing at differential genetic influences contributing to ADHD and SUD (Carpentier et al., 2013) and suggest that comorbidities should be considered in future genetic studies to identify reliable clinical subtypes, generate more homogeneous datasets and, eventually, shed more light into the role of genetic factors in ADHD (Buitelaar, 2005; Levy and Ebstein, 2009). Alternatively, discrepancies between ADHD with and without comorbid SUD could also be attributed to limited statistical power (40.2% considering a log-additive model of inheritance), additional genetic risk factors or differences in the frequency of other comorbid disorders co-occurring with ADHD and not considered in the present study.

Second, the association between the miR-183-96-182 cluster and ADHD should be considered preliminary until replication. In this regard, we have explored results from a meta-analysis of four previous genome-wide association studies in childhood ADHD, including 2064 trios, 896 cases and 2455 controls, and observed no statistically significant effects of the two SNPs at the 3′ region of miR-96 (rs2402959 and rs6965643) on the risk of developing ADHD (p = 0.75) (Neale et al., 2010). These results are consistent with the lack of association between the miR-183-96-182 cluster and the overall ADHD sample identified in our study. Because SUD data are not available in the childhood ADHD sample, follow-up studies of these patients may allow us to discern between individuals with and without symptomatic remission and/or comorbid SUD and, thus, to evaluate the involvement of the rs2402959 and rs6965643 in the subgroup of ADHD patients without comorbid SUD.

Third, because putative functional variants were not prioritized in the miRNA-96 SNP selection and the identified SNPs are located in the 3′ flanking region of the miRNA cluster, the risk haplotype may have no functional consequences by itself. Rather, it may be in linkage disequilibrium with other functional variants that would be the ones directly involved in the genetic vulnerability to ADHD. Indeed, in silico predictions showed that none of the SNPs conforming the ADHD risk haplotype would have relevant functional effects. Unfortunately, two sequence variants located within the miR-96 precursor (rs73159662 and rs41274239), and thus with a potential direct effect in miRNA biogenesis and function, were not considered in the present study due to their low MAF values (Sun et al., 2009).

Fourth, the relatively small sample size may provide imprecise estimates of the magnitude of the observed effects and may also contribute to the lack of association found between ADHD with comorbid disruptive behavior disorders (CD and/or ODD) and the functional polymorphism in HTR1B that was previously associated with aggressive behaviors (Conner et al., 2010; Jensen et al., 2009). Since we used DSM-IV diagnostic criteria for CD or ODD in a clinical dataset rather than self-report of anger and hostility used in previous studies, discrepancies could also be attributed to clinical heterogeneity and measurements of aggression-related behaviors. And finally, although population stratification in our sample was previously tested to reduce the likelihood of type I errors (Ribases et al., 2008, 2009), we cannot overlook that the association between ADHD and miR-183-96-182 did not remain significant after adjusting for multiple testing using the Bonferroni correction. This method, however, requires independence between all performed tests and, therefore, may be too restrictive since some of the selected SNPs show some degree of LD.

In summary, although the functional sequence variants in the miR-183-96-182 cluster directly involved in ADHD remain unknown, we have identified a tentative association between this genomic region and ADHD without comorbid SUD. These results strengthen the hypothesis that SNPs modulating the expression of miRNA or their interactions with target miRNAs may impact the development and maintenance of a specific transcriptome and thus contribute to the genetic susceptibility to complex disorders such as ADHD. Furthermore, these results emphasize the need to take comorbidities into account in genetic studies to minimize the effect of heterogeneity and to identify genetic variants associated with a higher or lower risk of developing drug addiction in ADHD patients. This information could help to clarify these complex phenotypes and provide clues to the underlying biochemical pathways, information about predictive risk factors involved in the development of SUD later in life and more insight into the underlying disease mechanism.

Role of funding source

MR is a recipient of a Miguel de Servet contract from the “Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación”, Spain, and CT was supported by the European Union (Marie Curie, PIEF-GA-2009-254930).


Contributors

Cristina Sánchez-Mora participated in the DNA isolation and genotyping assay design, undertook the statistical analyses and wrote the first draft of the manuscript.

Josep Antoni Ramos-Quiróga, Carlos Roncero, Rosa Bosch and Miquel Casas participated in the study design, clinical assessment and coordination of the clinical research.

Vanessa Richarte, Gloria Palomar, Mariana Nogueira, Montse Corrales, Constanza Daigre, Nieves Martínez-Luna and Lara...
Grau-Lopez participated in the clinical assessment and in the recruitment of patients.

Noelia Fernandez-Castillo, Iris Garcia-Martinez and Claudio Toma participated in the genotyping assay and the statistical analyses.

Bru Cormand and Marta Ribasés wrote the protocol, coordinated the genetic study design and statistical analysis and supervised the manuscript preparation.

All authors contributed to and have approved the final manuscript.

Conflict of interest

None of the authors have conflict of interests or relevant financial interests or personal affiliations in connection with the content of this manuscript.

Acknowledgments

We are grateful to all patients and controls for their participation in the study and to M.D. Castellà, B. Olivares, M. López and A. Daví for their help in the recruitment of control subjects.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.euroneuro.2013.07.002.

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