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Association analysis of norepinephrine transporter polymorphisms and methylphenidate response in ADHD patients

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ABSTRACT

Aims: Methylphenidate (MPH) is the most frequently prescribed drug in Attention Deficit Hyperactivity Disorder (ADHD). Hitherto mostly the dopamine transporter gene has been studied in MPH-response and only a few studies analyzed the norepinephrine transporter (*NET*, *SLC6A2*) gene, although MPH is a potent inhibitor of both dopamine and norepinephrine transporters. We aimed to analyze this monoamine transporter gene in relation to ADHD per se and MPH-response in particular to gain further knowledge in ADHD pharmacogenetics using a Caucasian sample.

Methods: Six single nucleotide polymorphisms (rs28386840, rs2242446, rs3785143, rs3785157, rs5569, rs7194256 SNP) were studied across the *NET* gene in 163 ADHD children (age: 9.3 ± 2.6; 86.5% male) using ADHD-RS hyperactivity-impulsivity and inattention scales. For case-control analysis 486 control subjects were also genotyped. At the MPH-response analysis responders had minimum 25% decrease of ADHD-RS total score after 2 months of treatment, and chi-square test compared 90 responders and 32 non-responders, whereas ANOVA was used to assess symptom improvement after the first month among the 122 ADHD patients.

Results: The classical case-control analysis did not yield any association with ADHD diagnosis, which was supported by meta-analysis conducted on the available genetic data (combining previously published and the present studies). On the other hand, the intronic rs3785143 showed nominal association with inattention symptoms ($p = 0.01$). The haplotype analysis supported this association, and indicated the importance of the first haploblock encompassing the intronic and 2 promoter SNPs. With MPH-response only the promoter rs28386840 showed nominal association: Those with at least one T-allele were overrepresented in the responder group (42% vs 19%, $p = 0.08$), and they had better improvement on the hyperactivity-impulsivity scale compared to the AA genotype ($p = 0.04$).

Conclusion: Although none of our single SNP findings remained significant after correcting for multiple testing, our results from the MPH-response analysis indicate the potential importance of promoter variants in the *NET* gene.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent childhood-onset psychiatric disorders, affecting 5% of school-age children worldwide (Polanczyk et al., 2007). The genetic component of ADHD has been demonstrated by family- and twin-studies, but genome-wide association studies have not yielded any main, ADHD-specific genetic locus, only candidate gene studies showed small but significant effect at a number of monoaminergic polymorphisms (Bonvicini et al., 2016; Gizer et al., 2009). Genetic factors determining drug responsiveness have been also intensively studied in the last decade, mostly in connection with methylphenidate (MPH) response in ADHD treatment (Froehlich et al., 2010), because only 65–70% of ADHD patients benefit from MPH treatment (Biederman and Spencer, 2008). The dopamine and norepinephrine transporter genes have been in the center of these studies, because MPH can compete with both catecholamines at their transporter binding, while it does not inhibit the serotonin transporter (Gatley et al., 1996; Han and Gu, 2006;

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Markowitz et al., 2006). Furthermore, an in vivo study showed that the usual clinical dose of orally administered MPH produces 70–80% inhibition of norepinephrine transporter (NET, *SLC6A2*) in humans (Hannestad et al., 2010).

Genetic association studies using single nucleotide polymorphisms (SNPs) of the *NET* gene have been controversial both in terms of ADHD diagnosis and MPH-response in ADHD patients. Although association studies pointed repeatedly to intronic variants at the first half of the *NET* gene (rs3785143 from intron 1 and rs11568324 from intron 5) in ADHD (Brookes et al., 2006; Kim et al., 2008), not all subsequent studies could replicate these associations (Hawi et al., 2013; Tzang et al., 2014; Xu et al., 2008). Therefore, further association studies using independent patient populations are still important. Our aim was to carry out case-control and dimensional association analyses of *NET* gene variants (located both on the 5′ and 3′ end) in ADHD patients. Meta-analysis was planned to support our single SNP analysis results, and haplotype analysis was carried out to assess further details of *NET* SNPs in ADHD.

Among the *NET* polymorphisms, the rs11568324 minor allele has a potential protective effect. However, the frequency of this allele is below 2%, therefore, we did not include this SNP in our analysis, and chose instead rs3785157 (from intron 7), which was previously indicated in ADHD by different research groups (Bobb et al., 2005; Hawi et al., 2013; Hohmann et al., 2015; Xu et al., 2005). The other selected *NET* polymorphisms included widely investigated SNPs, namely the intronic rs3785143 and exon 9 rs5569 (1287 A/G SNP). To cover the 5′ and 3′ non-coding gene regions of the *NET* gene we selected possibly functional gene variants. From the promoter region the rs2242446 (− 182C/T SNP) and rs28386840 (− 3081 A/T SNP) were chosen, because of their potential or proven influence on gene transcriptional activity (Kim et al., 2006; Sigurdardottir et al., 2016; Zill et al., 2002). From the 3′ end we selected a SNP (rs7194256) which has been shown to affect gene expression in an in vitro luciferase reporter gene assay (Marques et al., 2017). We hypothesized that either the 5′ or 3′ non-coding region's functional genetic variant(s) would have bigger effect compared to the intronic or synonymous polymorphisms in the NET gene.

Beside the association analyses of the *NET* genetic variants with ADHD diagnosis and symptoms, we aimed to characterize MPH-response in our ADHD patient sample. Hitherto, the *NET* pharmacogenetic findings have been mixed. The first pharmacogenetic study of the *NET* gene indicated better improvement on hyperactivity-impulsivity score (but not on inattentive scores) in rs5569 G-allele carriers among Chinese Han youths (Yang et al., 2004). Among the subsequent studies conducted in Korean populations two supported the better response of the GG genotype (Park et al., 2012a; Park et al., 2012b; Song et al., 2011), but other two studies did not (Lee et al., 2011; Kim et al., 2010). In addition, an American (double-blind, placebo-controlled) study did not find association between the rs5569 and MPH-response (McGough et al., 2009). However, a detailed genetic analysis in an open-label study of a MPH transdermal system indicated two other *NET* SNPs (rs17841329 and rs192303) from intron 1 (Mick et al., 2008). Concerning the promoter variants, two Korean studies showed better MPH-response at subjects with at least one rs28386840 (− 3081) T-allele. One of the studies showed better improvement at the Clinical Global Impression-Improvement score (Kim et al., 2010), and the other showed greater decrease in the mean commission error scores in a continuous performance test (Park et al., 2012b). Our pharmacological study was similar to these Asian ones, it was a prospective study using symptom severity scores assessed each month after starting an MPH treatment.

2. Methods

2.1. Subjects and clinical assessments

The study was designed in compliance with the Helsinki Declaration and was approved by the Local Scientific and Research Ethics Committee of the Medical Research Council. Patients and their parents provided written informed consent for their participation. Children with IQ < 80 (estimated from the Raven progressive matrices test; Raven, 1965), as well as those who had severe medical or neurological conditions, or pervasive psychiatric disorder were excluded from the study. Among the selected 163 children (mean age: 9.3 ± 2.6; 86.5% male) diagnosed with ADHD according to DSM-IV criteria (American Psychiatric Association, 1994) by two independent child psychiatrists, 122 children (mean age: 9.6 ± 2.6; 88.5% male) participated in a prospective MPH-response analysis. Comorbid conditions were assessed with the Hungarian child version of the Mini-International Neuropsychiatric Interview (MINI-Kid; Balazs et al., 2004), for detailed demographic and clinical characteristics see Supplementary Table 1.

The MPH-response criteria has been published previously (Kereszturi et al., 2008), in short, creating the category of non-responders was based on < 10% decrease in the total score of the ADHD Rating Scale (ADHD-RS, DuPaul, 1998) after 2 months of treatment (all of the non-responders discontinued the treatment after 3 months). The responders had at least 25% decrease in the ADHD-RS total score after 2 months of treatment; also their symptoms reduced to minimal after 5 months of treatment as measured by the Severity of Illness subscale of the Clinical Global Impression scale (CGI-S; Guy, 1976, point 1–2 corresponding to no or minimal symptoms) at the follow-up visits after 5 and 6 months of treatment. According to these criteria, 90 children were categorized as responder, whereas 32 children as non-responder. Patients participating in the drug response study were given 10–30 mg methylphenidate (Ritalin 10 mg, immediate-release) according to their body weight, in two doses (morning and noon). The daily dose thereby ranged from 0.22 to 0.95 mg/kg/day, in average 0.55 ± 0.15 mg/kg/day. Patients did not receive any other psychoactive medication.

For case-control analyses two control samples were used: 400 sex-matched healthy young adults were selected from the general population (mostly university students, see Varga et al., 2012) who did not report psychiatric disorder diagnosis in their lifetime on a self-report questionnaire. Additionally, a smaller group of children was tested (n = 86, 58.1% male, aged 6–7 years), which was screened for psychiatric diagnoses and also specifically for ADHD symptoms with the Hungarian version of the Child Behavior Checklist (Achenbach, 1991; Gadoros, 1996) rated by the mother as part of a longitudinal study (Birkas et al., 2006). Both the clinical and control samples were ethnically homogenous and of Caucasian origin (based on both biological parents' Hungarian origin), and consisted of unrelated individuals.

2.2. Isolation of DNA and genotyping

Genomic DNA was isolated from buccal cells by the DNA purification kit obtained from Gentra (Minneapolis, USA). The SNPs were genotyped with pre-designed TaqMan probes (rs28386840: C_60398891_10, rs2242446: C_26354911_10, rs3785143: C_27481932_10, rs3785157: C_27481947_10, rs5569: C__3020068_10, rs7194256: C_29079520_10) on 7300 Real-Time PCR System (Applied BioSystem, Foster City, USA). No significant deviations from Hardy–Weinberg equilibrium (p > 0.05) were detected for any of the polymorphisms either in the case or in the control groups.

2.3. Association analyses

In the categorical analyses chi-square tests were carried out, and p-value threshold for multiple comparisons was calculated by the False Discovery Rate adjustment (Benjamini et al., 2001), setting the significance threshold to $p < 0.008$. In the quantitative analyses MPH-effect was assessed with the ADHD-RS Inattention and Hyperactivity/Impulsivity score differences after the first month by analysis of variance. Haplotype frequencies were checked with the Haploview program (Barrett et al., 2005) and quantitative analyses of estimated