

The association between TaqI A polymorphism of ANKK1 (DRD2) gene and ADHD in the Czech boys aged between 6 and 13 years

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Abstract

OBJECTIVE: The purpose of this study was the correlation of the combined type of ADHD in children and Taq IA polymorphism DRD2 gene. We hypothesized a positive correlation of DRD2 polymorphisms in the combined type of ADHD patients without co-morbidity.

PATIENTS AND METHODS: Our research sample included 586 unrelated boys of the Czech origin aged between 6 and 13 years. The ADHD group consisted of 269 boys and the control group consisted of 317 boys. PCR detection of the DRD2 polymorphism was carried out by using primers, described by Grandy (Grandy *et al.* 1989).

RESULTS: The comparison of genotype frequencies showed statistically highly significant difference between the studied groups ($p < 0.0001$). A statistically significant difference was also found when the allelic frequencies between the two groups were compared ($p < 0.0001$), with the A1 allele having a 4.359 fold higher risk of ADHD (Risk Ratio=4.359, 95% CI of RR=3.5753 to 5.3144, Odds Ratio=7.7824; 95% CI of OR=10.315 to 13.6719).

CONCLUSIONS: Our results presented a highly positive correlation between the combined type of ADHD without co-morbidity and ANKK1 (DRD2) polymorphism.

INTRODUCTION

ADHD (Attention Deficit Hyperactivity Disorder) is a very frequently diagnosed syndrome in child psychiatry. Its prevalence is estimated at 3% to 6% of the child population; with boys outnumbering girls at 3:1 or higher ratio (Anderson *et al.* 1987). The prevalence is estimated at 5.23% (Polanczyk

et al. 2007) to 8%–12% (Biederman and Faraone 2005) in more recent studies. Significant differences in ADHD prevalence occur particularly between North America and Europe on one side and Asia and South America on the other side (review Polanczyk and Jensen 2008).

The key ADHD symptoms – inattention, impulsivity and hyperactivity negatively affect children's relationships, both in the family and with their contemporaries which increases the risk of a social isolation. Hyperkinetic disorder (ICD-10 – International Classification of Disorders, WHO 1992) is a narrower diagnosis and a subgroup of ADHD DSMIV diagnostic criteria, APA (American Psychiatric Association) 1994. Co-morbid disorders of ADHD occur in 50% to 80% of patients (Jensen *et al.* 1997). These include: conduct disorders in 40% to 90%, depressive disorders in 15% to 20%, anxiety disorders in 25% and learning disorders: dyslexia, dyscalculia etc. in 20% of all ADHD patients' cases.

The hyperkinetic syndrome is up to five times more frequent in first-degree relatives of ADHD children than in the control group of healthy children's families (Berman *et al.* 1997). Other diagnostic units were more frequent (co-morbid ADHD disorders) in the ADHD children's relatives than in the control group. When the occurrence of hyperkinetic disorder in biologically related and unrelated siblings of ADHD patients was compared, the following results were obtained: hyperactivity and conduct disorders were found in 47% to 53% of biologically related children and in 9% to 13% of biologically unrelated children (Safer 1973). A significant role of genetic factors was proven in adoption studies of ADHD children (Van Der Oord *et al.* 1994). Concordance rates of monozygotic vs. dizygotic hyperkinetic pairs of twins were between 50% and 80% for monozygotic twins and between 0% and 33% for dizygotic twins (Hechtman *et al.* 1994; Spencer *et al.* 1996; Thapar *et al.* 1995; 1999; Faraone *et al.* 2003; 2005). The heritability of ADHD fluctuates between 0.75 and 0.98, which is highly significant for genetic etiology of ADHD (Edelbrock *et al.* 1995; Faraone *et al.* 2003; 2005).

At present, an association of ADHD with more than forty dopaminergic, noradrenergic, serotonergic and other genes has been described, including some neurochemical data, that participate on ADHD endophenotypes definitions (Faraone 2005; Paclt *et al.* 2005; Durston *et al.* 2007; Faraone *et al.* 2006; Mick and Faraone 2008; Durston 2008). The strongest association with ADHD show: DRD4, DRD5, DBH dopamine transporter gene, SNAP 25, serotonin transporter gene, serotonin 1B receptor gene.

A question is whether DRD2 polymorphisms are relevant to ADHD etiology research. Some results were contradictory or negative (review Mick and Faraone 2008), but some new results of positive correlations of ADHD and DRD2 were published in the last two years.

Some basic theoretical and clinical data from experimental, biochemical and neuroimaging research were published. The absence of DRD2 polymorphism in experimental mice changed their locomotion, extremely increased their hyperactivity and reward behavior – animal model of ADHD (Maldonado *et al.* 1997). The HVA in CSF rises after stimulants adminis-

tration (Volkow *et al.* 1995). The level of HVA in CSF rises together with increasing density of the DRD2 receptors as a response to stimulants (Comings 1995; 1997). A relation between the DRD2 A1 genotype and the CNS blood flow was described in ADHD children. By using F-deoxyglucose in PET examination was discovered that the DRD2 A1 allele carriers significantly reduce glucose metabolism in the putamen, frontal, temporal, central, central, prefrontal, occipitotemporal and orbital cortex (Comings 1997; Tannock 1998). These structures are important in etiology of ADHD in children (review Durston 2008).

In studies of DRD2 gene and ADHD associations Comings *et al.* 1991 compared 104 ADHD subjects (nearly all TS co-morbid) with controls and discovered a significant association with Taq IA1 allele of DRD2. The genotype and the allelic frequency of TaqI A polymorphism of DRD2 gene were statistically different between the ADHD group of boys only (n=49) aged 6–13 and the control group (n=40) age 6–13, boys only $p<0.004$ and $p<0.001$, respectively (Šerý *et al.* 2003). 178 ADHD boys (aged 7–13) were examined in another independent study (Šerý *et al.* 2006; Drtílková *et al.* 2008) and compared with the control group of 108 unrelated healthy boys. DRD2 correlated with ADHD ($p<0.08$, $p<0.02$). More recently this finding was replicated in a case-control study (Kopečková *et al.* 2008): 100 children with the combined type of ADHD were examined and the results compared with the results of 100 children from the control group (all children aged between 6–10 years). The results of this study refer to the association of the polymorphisms in genes DRD2, 5HTT, DAT1 and DBH with ADHD. The risk of ADHD is significantly higher in homozygous with both mentioned alleles (54.8× in DRD2 gene). Nyman *et al.* (2007) used population sample birth cohort and identified positive association in DBH polymorphism and in DRD2. He compared 188 ADHD children patients with 166 control patients, 228 males and 128 females. A genetically isolated population group was used for the study. Berman *et al.* (1997) reported that an association of DRD2 A1 stress symptoms A2 allele with given phenotype was dependent upon the presence or absence of childhood stress symptoms. But Qiujin *et al.* (2007) did not confirm the association between DRD2 and ADHD in 340 Chinese ADHD children and 226 unrelated people from the control group.

Carrying out a family study, Rowe *et al.* (1999) examined 164 children from 125 families and found no excess transmission of the Taq 1 A1 allele. Todd *et al.* (2002) did not confirm the correlation of DRD2 receptor and ADHD.

New ADHD susceptibility genes 600.000 SNP were identified in a genotype family study – genotyped in a ADHD family probands. Parental trios with the 909 screening algorithm were examined SNP rs 6565 113 and rs 5525 55 that met the criteria for significance within a specified phenotype. 17 out of the 37 ADHD

candidate genes had associations p -value lower than 0.01, SCC GA1, SLC6A1, HES1, ADRB2, HTR1E, DDC, ADRA1A, DBH, DRD2, BDNF, TPH2, HTR2A, SLC6A2, PER1, CHRNA4, SNAP25 and COMT gene (Lasky-Sue *et al.* 2008.) Families were recruited via the combined type of ADHD child probands ($n=647$), who had at least one full sibling and one biological parent ($n=1227$) during the IMAGE study (Brookes *et al.* 2006). No significant associations were identified, but a trend towards significance $p=0.07$ of the Ser3 DRD2 polymorphism, when Ser311 polymorphism is paternally transmitted.

METHODS

The project was approved by the Ethic Committee of the Faculty Hospital Brno, Ethic Committee of the Faculty Hospital Prague and granted by the Internal Grant Agency of the Czech Ministry of Health. 586 unrelated boys of the Czech origin aged between 6–13 years were examined and observed in this study. The ADHD group consisted of 269 boys; the control group consisted of 317 boys. Informed consent was signed by legal guardians of all boys prior their involvement in the project. The diagnosis of ADHD was assigned in accordance with criteria of DSM-IV and the type of hyperkinetic disease was diagnosed according to ICD10 and DSM IV combined type. ADHD patients were selected mainly from the Faculty Hospital Brno Department of Child Psychiatry, as well as from the Children's Psychiatric Hospital Velká Bíteš and from the Charles University 1st Faculty of Medicine Department of Child Psychiatry Children's Psychiatric Center. Excluding criteria for the ADHD group were: the presence of inborn genetic defects, epilepsy, mental retardation, schizophrenia, pervasive developmental disorders, language and learning disorders, conduct disorder and somatic diseases. The control group consisted of primary school boys of corresponding age from Brno and Prague regions and partly of boys treated in the Faculty Hospital Brno for irrelevant somatic diagnosis. CPQ questionnaires (Parent Rating Scale, Conners 1985) were filled out by parents and CTQ questionnaires (Teacher Rating Scale, Conners 1985) were filled out by teachers prior to each boy's inclusion into the control group. Only hyperactivity/impulsivity factors were included in the point measuring of ADHD symptoms within the control group. Only boys with under 9 points in their CPQ questionnaire and under 7 points in the CTQ questionnaire were included into the control group. Hereby these questionnaires eliminated the inclusion of boys with ADHD symptoms into the control group.

Samples of buccal tissues were collected after the diagnosis was established. Sterile cotton swabs and DNA storage kits (EliDNA Store Kit, ELISABETH PHARMACON) were used for the collection of buccal samples. DNA in the collected samples remains stable for a few days at room temperature. DNA from swabs was

isolated by using the UltraClean Tissue DNA (MoBio, USA) commercial kit. Storage buffer with swab from EliDNA Store Kit was filled directly into columns and centrifuged. Columns were then washed by 400 microliters of TD2 buffer and DNA was eluted in TD3 buffer from UltraClean Tissue DNA Kit. The whole process of DNA isolation takes 10 minutes. Using this procedure, it was possible to take samples in schools without traumatizing the children by collecting blood samples.

PCR detection of the DRD2 polymorphism was performed using primers, described by Grandy (Grandy *et al.* 1989). The primer sequences were 5'- CCG TCG ACC CTT CCT GAG TGT CAT CA -3' and 5'- CCG TCG ACG GCT GGC CAA GTT GTC TA -3'. Amplification reactions were carried out in a volume of 50 μ l, containing 100 ng genomic DNA, 200 μ M each dNTP, 1 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 2 μ M of each of primers and 2.5 units of Taq-Purple DNA polymerase (Top-Bio). After initial denaturation at 94 °C for 1 min, DNA was amplified in three-step cycles as follows: denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s, using the TouchgeneGradient thermal cycler (Techne, England). After 35 cycles, a final extension time of 5 min was applied at 72 °C. The length of the amplified fragment was 318 bp. The PCR products were analysed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under UV light. The PCR products were digested with 8 units of TaqI restriction enzyme (New England BioLabs, England) at 65 °C for 5 h. Digestion products were analysed by agarose gel electrophoresis as described above. The amplified fragment is cleaved by TaqI restrictase to 308 bp and two 6 bp and 4 bp long fragments. The 308 bp long fragment can be cleaved to two 180 bp and 128 bp long fragments. The uncut product of 308 bp identified A1/A1 genotype, the A1/A2 genotype was characterized by three fragments 308 bp, 180 bp and 128 bp, and the A2/A2 genotype was indicated by two fragments 180 bp and 128 bp.

Statistical analysis

CSS Statistica Software (StatSoft, USA) was used for the results statistical evaluation. Contingency tables were constructed from the results of genotyping. The chi-square test was used for the genotype frequencies comparison in the studied groups; Fisher's exact test was used for of the allelic frequencies comparison.

RESULTS

The results of the rs1800497 polymorphism genotyping are summarized here: 45 of the patients with ADHD carried the A1A1 genotype, 104 of them were heterozygotes A1A2 and 120 were A2A2 homozygotes. 6 individuals in the control group were A1A1 homozygotes, 81 were A1A2 heterozygotes and 230 were A2A2 homozygotes.

Comparison of genotype frequencies showed statistically highly significant difference between the studied groups ($p < 0.0001$). A statistically significant difference was also found when the allelic frequencies between the two groups were compared ($p < 0.0001$), with the A1 allele having a 4.359 fold higher risk of ADHD (Risk Ratio=4.359, 95% CI of RR=3.5753 to 5.3144, Odds Ratio=7.7824; 95% CI of OR=10.315 to 13.6719).

DISCUSSION

The results of the ADHD genetic research may be influenced by many factors. It is often a question of different, geographically related, diagnostic methods described in various studies (Polaczyk and Jensen 2008).

Although ADHD is a highly heritable condition (Thapar *et al.* 1999; Faraone *et al.* 2005), none of the investigated genes has proven to be either sufficient or indispensable for causing the disorder. As for all complex traits conditions, the etiology of ADHD is thought to involve a combination of multiple genes of moderate effect (Thapar *et al.* 1999). There are some other possible etiological factors, especially the participating or dominating role of ADHD prenatal etiology (hypoxia in gravidity, alcohol abuse, drug abuse or smoking in the gravidity). There are very few studies that excluded these etiology factors.

The question is whether there may be some differences in the genetics of various ADHD type by DSMIV (attention type, combined type, hyperactivity/impulsivity type). Samples of children only with the combined type of ADHD (Chen *et al.* 2008) are optimal for these studies.

Some data in recent studies correlating ADHD and DRD2 genes polymorphism were inconclusive for several reasons, e.g. *age factor* – children, adolescents and adults or *co-morbidity with conduct disorder* (Kim *et al.* 2006). Developmental changes in ADHD psychopathology were documented in many previous studies (review Sonuga and Barke *et al.* 2008). The relationship between CD and ADHD is still unclear from the phenotype (Neale and Faraone 2008) or genotype (Anney *et al.* 2008) point of view. From the results of various studies of ADHD, co-morbid ODD and CD is possible that there are some mistakes in the ADHD identification. The data obtained in these studies of co-morbidities ODD, CD and ADHD remain currently unclear (Anney *et al.* 2008). It would be necessary to study separately children with CD or ODD co-morbidity with ADHD and without this co-morbidity for a successful and relevant methodology.

It is difficult in various family genetic studies to fully exactly *describe* some groups of the experimental persons because their parents with prominent symptoms of ADHD or/and CD, personal disorder in adulthood refuse to take part in the studies. This is very important for the final results, because when the parents

with prominent ADHD or CD problems refuse genetic examination, these complications may change results of the study.

There are a few positive results for association of DRD2 and ADHD in the family studies of the candidate polymorphisms genes, but there are more positive results and trends in the family genomic studies (Lasky-Sue *et al.* 2008, Brookes *et al.* 2006).

The above described sample from this study included 586 unrelated boys of the Czech origin, aged between 6–13 years. The ADHD group consisted of 269 boys and the control group consisted of 317 boys. A comparison of the genotype frequencies showed statistically highly significant difference between the studied groups ($p < 0.0001$). A statistically significant difference was also found when the allelic frequencies between the two groups were compared ($p < 0.0001$). Our case-control study was carried out in children with the combined type of ADHD without co-morbidity and will be followed by a family study.

At present more positive DRD2 research results of correlation of DRD2 and ADHD like co-morbidity of addictive disorders have been published. Najafapady (*et al.* 2005) published a study of 100 opium addicted Iranian patients and 130 unrelated people in the control group: when associated A1 and A2 allele of DRD2 and ADHD, a significant association was observed only between A1 allele and ADHD opium addicted patients ($p < 0.0001$). The frequency of A1 DRD2 genotype was significantly higher in patients compared with individuals in the control group ($p < 0.0001$). ADHD is often accompanied by an increased risk of smoking. The analysed sample included 1900 adolescents with identified genotype data. Multiple logistic regressions were used to examine the relationship between cigarette smoking and ADHD (retrospective report). DRD2 predicts smoking status ($p = 0.04$) in the ADHD group and it is necessary to have at least 6 positive ADHD retrospective symptoms for a positive correlation. Ballon *et al.* (2007) found out that Wender's Utah Rating Scale presented positive association of DRD2 and/or DRD4 polymorphisms in the group of patients with childhood ADHD or in a group of cocaine addicted patients with high impulsivity score in retrospective study (sample of 97 men). These results document the possibility of a higher risks for ADHD children in contact with some drugs. Study of these topics is often positive only in correlation of DRD2 and ADHD and co-morbid drug addictive disorder, not in other addictive patients with ADHD.

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