Lack of association of polymorphisms in six candidate genes in colombian adhd patients

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ABSTRACT

Background: Attention Deficit and Hyperactivity Disorder (ADHD) is a common childhood neuropsychiatric condition. The disorder has a multifactorial background, with heritability estimates of around 76%, suggesting an important role of genetic factors. Candidate genes include those related to dopaminergic (e.g. DRD4, DRD5, SLC6A3 and DBH) and serotoninergic (e.g. HTR1B and SLC6A4) pathways.

Purpose: To explore the association of common polymorphisms in six genes (DRD4, DRD5, SLC6A3, DBH, HTR1B and SLC6A4) and the susceptibility to ADHD in a Colombian sample population.

Methods: Eighty-six ADHD trios and 152 healthy controls were recruited. Genotyping of the six polymorphisms was performed using described PCR-based protocols. A TDT analysis was used to test if there was preferential allelic transmission for any of the six polymorphisms. Additionally, a case-control analysis was performed to test for association of the serotoninergic (HTR1B and SLC6A4) polymorphisms with ADHD.

Results: Through the TDT analysis there was no preferential allelic transmission for any of the studied variants. Case-control analysis did not show association.

Conclusion: This is the first study in Latin America to describe six polymorphisms in a group of patients with ADHD. There was no evidence of association for any of the studied polymorphic variants in this Colombian ADHD sample. Further research, with larger sample sizes and study of endophenotypes, is needed in this population to confirm and extend the results.

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System for Children (BASC) were applied to parents and teachers of patient, in order to confirm the diagnosis. The Wechsler scale of intelligence (WISC-R) was applied in order to exclude children with a score less than 70, which indicate an intellectual disability. Cases were selected among children who had at least one of the following criteria (applied to information derived from parents or teachers): 1) Six or more points, over nine, for inattention in the DSM-IV criteria and a percentile over 85 in the inattention domain of BASC; 2) Six or more points, over nine, for hyperactivity in the DSM-IV criteria and a percentile over 85 in the hyperactivity domain of BASC. The patient’s group included 86 children. Children who did not meet ADHD criteria, did not have intellectual disability (WISC-R >70) or other neurological disorders, were included in the study as controls (n = 56). In order to have a bigger sample size, an additional 96 DNA samples were included, among children or adults who did not have a history of ADHD or any other cognitive, neurological or psychiatric disorder. Finally, the control group included 152 subjects.

Peripheral blood or buccal samples were taken from all subjects. Parents of cases and controls approved and signed the informed consent after a detailed explanation. The Institutional Ethical Committee approved this study (Act 117, May 29th, 2008). To increase DNA samples, an amendment was presented to the ethical committee and approved by the act 165 of May 13th 2010.

**DNA extraction**

DNA extraction was carried out from peripheral blood or buccal samples using conventional techniques.

**Genotyping**

VNTRs in DRD4 and SLC6A3 as well as the insertion/deletion in the promoter region of SLC6A4 genes were genotyped by PCR followed by electrophoretic analysis as previously described. DNA amplification reactions were performed with GoTaq Green Master Mix (Promega), using the following cycling conditions: 94°C for 10 min, followed by 30 cycles of 94°C for 45 sec, 61°C for 45 sec, 72°C for 45 sec. Amplification was completed with a final elongation step of 72°C for 10 min. Fragments were separated on 1.2% agarose gels and visualized using ethidium bromide.

Polymorphisms in DBH (Taq1 A polymorphism) and HTR1B (rs6296) genes were genotyped by PCR-RFLP. For HTR1B the PCR product was digested with HincII restriction enzyme at 37°C overnight, and the PCR product of DBH was digested with Taq1 at 61°C for 16 hours. The alleles were detected after separation on an agarose gel. Experimental approaches were used as published before.

The dinucleotide (CA)n repeat polymorphism located in the DRD5 gene was genotyped using PCR with fluorescently labeled primers, followed by capillary electrophoresis in an ABI 3500 sequencer. Genotypes were analysed with Gene Mapper 4.1 software (Applied Biosystems).

**Statistical Analysis**

Statistical analysis was carried out by two different approaches. First, a transmission disequilibrium test (TDT) was done in 86 trios: corresponding to the index cases and their two biological parents. This analysis was used to test if there was preferential allelic transmission for any of the six polymorphisms. Genotype data was analyzed using PLINK. PLINK is a commonly used program for analysis of genetic studies, which is freely available and uses two main files as input: PED and MAP files.

A second complementary analysis was done through a case-control study where 73 cases and 152 controls were included. An analysis was performed for polymorphic variants in HTR1B and SLC6A4 genes (because these were found to be associated more frequently with the disorder), using PLINK and SNPStat software. Results for TDT and case control analysis were based in a X2 statistic and corrected for multiple testing with a Bonferroni correction.

**Results**

Cases were classified in subtypes according to DSM-IV criteria and BASC. Combined cases corresponded to 50.7%, 35.6% cases categorized as inattentive and 13.7% were hyperactive.

Genotype and allele frequencies are presented in Table 1. For VNTR in DRD4, alleles of two, four, seven and eight repeats were found. For the dinucleotide of DRD5 more than ten alleles of several molecular weights were found, but a predominant allele of 148 bp was evident. Therefore alleles were grouped into the allele with a molecular weight of 148 bp and others, named “No 148 bp”. Only two alleles were found for the VNTR of SLC6A3, corresponding to alleles of nine and ten repeats. In DBH gene, the Taq1 A polymorphism gives only two alleles (A1 and A2) distinguished by the presence or not of this restriction site, respectively. The alleles of the studied SNP in HTR1B corresponded to the presence of a cytosine or a guanine in the position 861 of the gene coding region. Finally, the alleles corresponding to the 44-bp insertion/deletion of SLC6A4 are called L and S (for large and short respectively) distinguished by a difference of plus (insertion) 44-bp or minus (deletion) 44-bp respectively. We found a larger than expected deletion (approximately 100 bp) in the promoter region of SLC6A4 gene in only one patient, which we determined as an atypical shorter allele, but no further analysis was implemented in this sample. All polymorphisms studied here were in Hardy Weinberg equilibrium (p>0.05). Figure 1 shows the electrophoretic gels for the polymorphisms studied through a PCR-based technique.

As TDT study only allows two alleles to be analyzed, the two most frequent alleles were considered for polymorphisms having more than two alleles. For example, for the VNTR of DRD4, the 4 and 7-repeats allele were considered as they were the most prevalent. Similarly, for SLC6A4 where three alleles were found, only L and S alleles were included in analysis based on their higher prevalence.

Association analysis through TDT did not demonstrate a preferential allelic transmission for any of the studied polymorphisms when ADHD or its subtypes were evaluated. This is probably due to a high homozygosity percentage for some variants, leading to exclusion of several trios and therefore leads to reduced sample size in TDT analysis (Table 2).

Case-control analysis for variants in HTR1B and SLC6A4 genes did not show evidence of association with ADHD susceptibility (Table 3).

**Discussion**

Although several works suggest an important influence of inherited factors in the development of ADHD, these genetic...
### Table 1: Allele and Genotype Frequencies

| Gene | Polymorphism (location) | Allele frequency | Genotype Frequency |
|------|-------------------------|------------------|--------------------|
|      |                         | Allele Proportion | Genotype Proportion |
| **DRD4** | VNTR (exon 3) | | |
| | 2 | 0.08 | 2/2 | 0.005 |
| | 4 | 0.69 | 2/4 | 0.112 |
| | 7 | 0.21 | 2/7 | 0.015 |
| | 8 | 0.02 | 2/8 | 0.005 |
| | 4/4 | 0.477 | 0 |
| | 4/7 | 0.29 | 0.7/ |
| | 4/8 | 0.005 | 0.091 |
| | 7/7 | 0.091 | 0.005 |
| | 7/8 | 0.005 | 0.005 |
| **DRD5** | Dinucleotide (CA)n (18,5 kb 5’ to the start site) | 148 bp No 148 bp | 148bp/148bp No 148bp No 148bp/No 148bp |
| | 0.395 | 0.605 | 0.140 |
| | 0.140 | 0.368 | 0.491 |
| **SLC6A3** | 40 bp VNTR (3’UTR) | 9, 10 | 9/9, 10/10 |
| | 0.208 | 0.792 | 0.071 |
| | 0.276 | 0.654 |
| **DBH** | TaqI A (intron 5) | A1, A2 | A1/A1, A1/A2, A2/A2 |
| | 0.302 | 0.698 | 0.052 |
| | 0.5 | 0.448 |
| **HTR1B** | rs6296 (coding region) | C, G | C/C, C/G, G/G |
| | 0.439 | 0.561 | 0.201 |
| | 0.476 | 0.323 |
| **SLC6A4** | 44 bp insertion/deletion (promoter) | L, S Atypical shorter allele (ASA) | S/S, S/L, ASA/L, L/L |
| | 0.509 | 0.488, 0.003 | 0.25 |
| | 0.476 | 0.006 |
| | 0.268 |

**Fig. 1:**
(A) Genotyping of VNTR located in exon 3 of *DRD4*. Lines 1 and 2: Heterozygote 2/4, Lines 3, 5, 6: Heterozygote 4/7, Line 4: Homozygote 4/4, Line 7: 50 bp ladder. (B) Genotyping of VNTR located in the 3’UTR of *SLC6A3*. Line 1: Homozygote 9/9, Line 2: Heterozygote, Line 3: Homozygote 10/10, Line 4: 50 bp ladder. (C) Genotyping of TaqIA polymorphism in *DBH*. Lines 1 and 2: Heterozygote, Line 3: 50 bp ladder, Line 4: homozygote A1/A1, Line 5: homozygote A2/A2. (D) Genotyping of SNP rs6296 of *HTR1B*. Line 1: Homozygote C/C, Lines 2 and 3: Homozygote G/G, Line 4: 100 bp ladder, Line 5: Negative control, Line 6: Heterozygote. (E) Genotyping of the 44-bp insertion/deletion in *SLC6A4*. Lines 1 and 5: Heterozygote, Lines 2 and 3: Homozygote S/S, Line 6: Homozygote L/L.
Table 2: TDT association analysis

| Gene    | A1 | A2 | T  | U  | OR  | CHISQ | p         | Exclusion % |
|---------|----|----|----|----|-----|-------|-----------|-------------|
| SLC6A3  | 9  | 10 | 17 | 24 | 0.708 | 1.195 | 0.2743    | 49.30%      |
| DBH     | A1 | A2 | 38 | 46 | 0.826 | 0.762 | 0.3827    | 30.10%      |
| DRD4    | 7  |   | 21 | 25 | 0.832 | 0.923 | 0.4531    | 28.13%      |
| DRD5    | No-148 | 148 | 32 | 33 | 0.922 | 0.015 | 0.9513    | 42.16%      |
| HTR1B   | C  | G  | 38 | 39 | 0.974 | 0.013 | 0.9093    | 16.20%      |
| SLC6A4  | S  | L  | 33 | 43 | 0.767 | 1.316 | 0.2513    | 22.90%      |

A1: Allele 1, A2: Allele 2, T: Transmitted, U: Untransmitted, OR: Odds-Ratio, CHISQ: Chi square, p: p value, Exclusion: Trio exclusion percentage.

Table 3: Allelic and Genotype Association using case-control approach

| Gene | MAF     | Allelic Association (p*) | Genotype Association (p*) |
|------|---------|--------------------------|--------------------------|
| HTR1B| 0.4231  | 0.2371                   | 0.2919                   |
| SLC6A4| 0.4686  | 0.689                    | 0.7071                   |

MAF: Minor Allele Frequency. p: p value. *Bonferroni correction.

studies involving candidate polymorphisms reveal that the genetic architecture of this disorder is extremely complex.5,32

Several epidemiological and statistical approaches, in populations of different genetic background, has led to controversial results.5

Despite the fact that several candidate genes have a biological role in to ADHD pathophysiology, some studies have failed to find a significant association.11,33,34 Our TDT study performed in the population of Colombian ADHD patients also failed to find a significant relationship with several candidate genes. Since we did not have previous data for homozygosity for these variants in Colombia population, we could not determine a percentage of trio exclusion before the study, which was as high as 49.3% for some genes (SLC6A3 for example). The high percentage of excluded trios led to small sample size for several variants. A complementary case control approach also failed to find a significant association in our samples.

As ADHD is a complex disease, it is possible that each single polymorphism has a relatively small contribution to the overall risk, but all variants together could underlie a large genetic influence.35 This hypothesis presupposes large sample size in order to find the relation of a specific variant with this disorder. Therefore a large sample of well-characterized patients is a challenge for research groups in developing countries.

Additionally, it is well known that ADHD is highly heterogeneous and has a clinical overlap with other neuropsychiatric conditions,16 making it difficult to associate a single variant with a single phenotype.35 The clinical heterogeneity of ADHD and other neuropsychiatric disorders has opened the door for the study of endophenotypes as a good approach in genetic studies. Endophenotypes refer to the analysis of specific behavioral symptoms, among others, as more stable and less heterogeneous phenotypes.5 Other studies from our group, for example, have shown the contribution of a polymorphism located in SLC6A3 (DAT1) gene to the development of an ADHD endophenotype in a Colombian sample.38 We have also previously found an association between two polymorphisms of SNPA25 and ADHD.39

Conclusions

Our study has failed to demonstrate an association of six polymorphic variants located in different candidate genes and the susceptibility to ADHD in Colombian population. Our findings are in concordance with results from genetic studies other populations.24,40 Future prospects in the genetic study of ADHD might include analysis of related endophenotypes.

Authorship contributions

Dora J Fonseca: Participated in the study design, data collection, analysis and interpretation of data and drafting the manuscript, Heidi E Mateus: Participated in the study design, data collection, analysis and interpretation of data, Jubby M Gálvez: Participated in the data collection, analysis and interpretation of data, drafting the manuscript and revising the final paper, Diego A Forero: Participated in study design, statistical analysis and interpretation of data, revising the final paper, Claudia T-Gutierrez: Participated in design, study supervision, statistical analysis and interpretation of data, as well as drafting the manuscript and revising the final paper.

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