ORIGINAL ARTICLE

Polymorphisms and low plasma activity of Dopamine-beta-hydroxylase in ADHD children

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Abstract

Attention-deficit Hyperactivity disorder (ADHD) is a multifactorial disorder clinically characterized by inattentiveness, impulsivity and hyperactivity. The occurrence of this disorder is between 3 and 6% of the children population, with boys predominating over girls at a ratio of 3:1 or more. The research of some candidate genes (DRD4, DAT, DRD5, DBH, 5HTT, HTR1B and SNAP25) brought consistent results confirming the heredity of ADHD syndromes. Dopamine-beta-hydroxylase (DBH) is an enzyme responsible for the conversion of dopamine into noradrenaline. Alteration of the dopamine/noradrenaline levels can result in hyperactivity. The DBH protein is released in response to stimulation. DBH activity, derived largely from sympathetic nerves, can be measured in human plasma. Patients with ADHD showed decreased activities of DBH in serum and urine. Low DBH levels correlate indirectly with the seriousness of the hyperkinetic syndrome in children [19,20]. In the DBH gene, the G444A, G910T, C1603T, C1912T, C-1021T, 5´-ins/del and TaqI polymorphisms occur frequently and may affect the function of gene products or modify gene expression and thus influence the progression of ADHD. This article reviews the DBH itself and polymorphisms in the DBH gene that influence the DBH activity in the serum and the CSF level of DBH. All those are evaluated in connection with ADHD.

Abbreviations

5HTT	– serotonin transporter
A	– alanine
Arg	– arginine
ADHD	– Attention–deficit Hyperactivity Disorder
cDNA	– complementary deoxyribonucleic acid
CSF	– cerebrospinal fluid
Cys	– cysteine
DAT	– dopamine transporter
DBH	– dopamine–beta–hydroxylase
DRD4 (DRD5)	– dopamine receptor D4 (D5)
DSM–IV	– The Diagnostic and Statistical Manual of Mental Disorder, 4th edition
h^2	– heritability
HTR1B	– 5–hydroxytryptamine (serotonin) receptor 1B
HWE	– Hardy–Weinberg equilibrium
ICD–10	– The International Clasification of Diseases, 10th edition
LD	– linkage disequilibrium
QTL	– quantitative–trait locus
S	– serine
SNAP25	– synaptosomal–associated protein
SNP	– single nucleotide polymorphism
STR	– short tandem repeat

Dopamine-beta-hydroxylase – biochemistry and physiology

The Dopamine-beta-hydroxylase (DBH) is an enzyme that catalyses the conversion of dopamine to noradrenaline. In its feedback, it inhibits tyrosine-hydroxylase, which reduces the production of dopamine. DBH is found in the brain, in catecholamine vesicules of the noradrenergic neurons of the gray matter in nerve terminals [32], in sympathetic nerves and in the adrenal medula, where it is present in both soluble and membrane-bound forms [51]. The DBH gene, which encodes DBH protein (OMIM 223360), was cloned, mapped to chromosome 9q34, and shown linked to ABO [8]. It is approximately 23 kb long, contains 12 exons coding 603 amino-acids protein [30] and exists as a single gene on genome. The genomic sequence is publicly available (Genbank accession numbers AC000404 and AC001227).

DBH is released into the circulation together with neurotransmitters and other vesicular content during synaptic transmissions [51,35] from sympathetic neurons and its enzymatic activity is analysed in plasma or serum [52] as an indicator of sympathetic noradrenergic tone. DBH found in the cerebrospinal fluid (CSF), predominantly originates in the central noradrenergic neurons [34], while DBH in the serum originates in the sympathetic nervous system [51]. Both forms correlate strongly with each other and are under a strong genetic control, with heritability of serum DBH estimated at 0.98 and that of CSF DBH at 0.83 [36]. DBH itself is the major quantitative-trait locus (QTL) for plasma DBH activity [12,55]. Associations with variation in both plasma DBH activity [50,12,11] and CSF levels of immunoreactive DBH protein [12] were shown. The difference in measured enzyme activity thus reflects differences in DBH protein level, rather than in homospecific activity (i.e., activity per mole of enzyme).

Plasma DBH activity varies widely across unrelated individuals [53]. However, developmental studies of noradrenergic trasmission during the ageing process are conflicting and the investigation of serum noradrenaline, especially in children, is very complicated for methodological reasons. The activity of noradrenergic system increases with age [58,17]. Paclt et al. [39] examined developmental changes of DBH plasma activity in relation to age in humans in a representative group of children and found that DBH activity rises continually with the exception of puberty period. It increases between 3–10 years of age and then decreases approximately at the age of 10–14 years. At the age of 21 to 60, the DBH level is stable. These findings were confirmed by experiments on animals (rats), showing the same developmental trend of enzymatic activity [39]. Weinshilboum and Axelord [52] did not find any differences in plasma DBH activity in male and female subjects. Suzuki et al. [46] described developmental changes of DBH activity in CSF of children, adolescents and adults. The results confirmed a continual rise of DBH activity and plasma DBH activity in children, with the exception of those aged 10 to 11.

Genetics of ADHD and dopamine-beta-hydroxylase gene

Attention-deficit Hyperactivity Disorder, ADHD, is one of the most common mental disorders that develop in children. The estimations of prevalence differ with the diagnostic criterion used. The Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV), which indicates between 3 and 6 percent of children with ADHD is the most common reference today. The International Clasification of Diseas, 10 th edition (ICD-10) is less strict and indicates 0.5 percent of afflicted children. A boys predominance over girls at a ratio 3:1 or more exists [1]. The principal characteristics of ADHD are inattention, hyperactivity and impulsivity. These symptoms appear early in a child's life, some of them persist to the adult age (approximately about 40–50% [38]), although they tend to diminish with age and social maturing. However, the relationships of these children both in the family and with their contemporaries are affected, increasing the risk of social isolation. 50–80 percent of ADHD children are afflicted with another co-morbid disorder [27], including oppositional defiant disorder and conduct disorder, anxiety disorder (25–30%), mood disorder (approximately 15%) and learning disabilities (between 20–30%) [4].

ADHD is a polygenetic disorder with various candidate genes. The multifactorial concept is consistent with high population prevalence of ADHD (3–6%), high concordance in monozygotic twins (68–81%), but modest recurrence risk to first-degree relatives [29]. It seems that ADHD is a complex genetic disorder, with many susceptibility genes with a small effect each [48]. Also, as the heritability (h^2) of ADHD is less than 1.0

(approximately 0.75–0.91) [32], it is likely that environmental factors also play a role in the causation and outcome of ADHD [29]. The exact mode of transmission is unknown. Various models of inheritance exist, from major gene effect to polygenic and multifactorial models [15,22], but the differences in statistical average between multifactirial genetic models and single gene inheritance are relatively small. The research of only some candidate genes (DRD4, DAT, DRD5, DBH, 5HTT, HTR1B and SNAP25) brought consistent results, confirming the heredity of ADHD syndromes [16].

It is likely that different neurotransmitter systems and the relative balance between them have varying degrees of influence over these behavioural dimensions. Variation in genes involved in these neurotransmitter systems are likely to mediate this delicate balance and have an effect on the function of these chemicals in the brain [40].

Genetics of dopamine-beta-hydroxylase activity

One of the important candidate genes is the DBH gene [16]. In experimental animals with decreased DBH in the serum, reduced conversion of dopamine to noradrenaline reduce the negative feedback on tyrosine-hydroxylase. These animals showed hyperactivity, aggression, self-stimulation and stereotypic movements [2,41]. The DBH gene therefore suggests hyperdopaminergic transmission in ADHD [29]. An association was made between different allelic variation at the DBH gene and both plasma DBH activity and CSF levels of DBH in many studies. Polymorphisms **G444A, G910T, C1603T, C1912T, C-1021T, 5´-ins/del** and **TaqI** exist.

G444A Cubells et al. [12] studied the relationship between genotypes at this synonymous polymorphism situated in exon 2 and CSF levels of DBH protein and plasma DBH activity. They observed a significant association between the G444A genotype and both biochemical phenotypes. Furthermore, investigation of European American patients with mood or anxiety disorders suggested that the 444A allele is associated with lower plasma DBH activity and the 444G allele with higher plasma activity. Their results support the hypothesis that DBH is a major locus influencing the plasma DBH activity and the CSF DBH protein levels. Although polymorphism G444A alters the third base of a Glu codon, the primary structure of DBH protein does not alter. The alterations of CSF levels of DBH protein and plasma DBH activity in coherence with this polymorphism may be explained by G444A residing at the splice junction between exon 2 and intron 2 of DBH. Kobayashi et al. [30] demonstrated that appropriately spliced mRNAs contain either G or A allele. Neverheless, this substitution could modify the efficiency of the mRNA splicing, thereby affecting levels of mature DBH mRNA and causing the differences in levels of DBH. Cubells et al. [11] also analysed linkage disequilibrium (LD) between G444A polymorphism and another plasma DBH-associated diallelic variant - 5´-ins/del- and confirmed their positive LD.

G910T This single nucleotide polymorphism (SNP) is non-synonymous and is located in exon 5. The difference at the nucleotide 910 causes an amino-acid altering between Ala (A) and Ser (S) at the amino-acid residue 304 [26]. Ishii et al [26] expressed the two gene variants in COS cells and suggested homospecific activities of DBH. The two forms of protein showed enzyme activities, immunoreactives, both of them had similar kinetic constants, but different homospecific activities. Ishii et al. [29] found a 13-fold diference in homospecific DBH activity between 910G and 910T alleles, with 910T encoding the lower homospecific active form. Zabetian et al. [55] examined A304S in groups of individuals representing phenotypic extremes with very low DBH activity levels. The samples were from African American, European American and Japanese population, but there were no deviations from the Hardy-Weinberg equilibrium (HWE) in these cases. Furthermore, Cubells and Zabetian [9] examined the potential functional consequences of A304S. They used the SIFT software to predict whether an amino-acid substitution affects protein function and established that A304S should be well tolerated. Interestingly, both alleles are presented in most human populations representing all major geographic regions, but only two population samples contained 910T (304S) homozygotes (Danes and Adygei). 910G (304A) is always a more common allele, with frequencies greater than 0.8 in every investigated group [10]. Further work will be necessary to evaluate the contribution of this polymorphism to heritable variation in the level and activity of DBH in serum and CSF and eventually to ADHD.

C1603T The other non-synonymous SNP is C1603T in exon 2, +1603 base pair from start site of translation. It encodes a non-conservative difference in the primary amino-acid sequence Arg535Cys and current results suggest that an allelic variance is responsible for a change in homospecifity of the enzyme [47]. Whereas plasma DBH activity is mostly influenced by the level of circulating DBH protein [11,35], in this case the 1603T allele (encoding 535Cys) appear to exhibit an additional effect due to decline in homospecific activity. The DBH holoenzyme is a homotetramer and Arg535Cys substitution may create origin disulfide bridge formation, thus altering the homospecific activity of DBH. Additionally, diferences in exocytotic release of DBH protein or in its clearence from the plasma [47] may occur. The research using the SIFT software predicted that SNP C1603T would be weakly tolerated and thus might affect DBH function, because Arg is conserved in all available sequences that include the 3´end of the gene [9]. Zabetian et al. [55] supported this prediction. They found a small but significant contribution to the variance of plasma DBH activity of this SNP. Tang et al. [47] confirmed previous results. They estimated the biological effect of C1603T on plasma DBH activity in a diagnostically heterogenous group of European population. In this sample, C-1021T genotype was found and it was confirmed that no significant LD between both polymorphisms existed.

They detected a significant additional effect of C1603T in the plasma DBH variance. C-1021T SNP accounts for 35–52 % of the variance in the trait across populations of different geographic origin [55], C1603T may explain additional 2% of variance. The low-activity 1603T allele is relative rare, with an occurrence of approximately 4% in the European population [55].

C1912T This SNP, located in exon 12, represents another member of the non-synonymous polymorphisms group. It changes the first nucleotide in codon for Arg, +1912 base pair from start site of translation, which leads to substitution of Arg->Cys. Arg->Cys should be weakly tolerated [9]. Exon 12 encoded the 3´-terminal region spanning from nucleotide +1681 to +2693 of type A cDNA and +1681 to +2393 of type B of DBH gene. Kobayashi et al. [30] examined both types of mRNA and showed evidence of inherence of two polyadenylation sites corresponding to types A and B in exon 12. The A and B types are produced by alternative polyadenylation.

C-1021T Zabetian et al. [55] sequenced a total of 6443 bp of DBH, including the proximal 1468 bp of the 5´upstream area, all exons and 2182 bp of intronic sequence in groups of individuals with very low, average and high plasma DBH activity to locate a new polymorphism associated with plasma DBH activity. Their experiments identified a C->T substitution located -1021 bp upstream of the translational start site, within the promotor, as an appropriate candidate (four from eight very low DBH activity individuals were TT homozygous). Subsequently, they examined C-1021T association to plasma activity in samples from African American, European American and Japanese population and showed the strong association of TT genotype with very low plasma DBH.

Further investigations led to the suggestion that C-1021T could be the major functional DBH polymorphism. The findings of other groups support this contemplation:

1) Dunnette and Weinshilboum [14] reported that DBH^{L} allele cause lower plasma DBH activity by diminishing the levels of circulating DBH protein, rather than by decreasing the activity of homospecific enzymes. C-1021T resides within the promotor and participates in the regulation of transcription.
2) Hoyle et al. [23] performed an experiment with human DBH gene in transgenic mice that suggests a region between –600 bp and –1100 bp contains elements fundamental for human DBH gene expresion in noradrenergic neurons.
3) Kim et al. [28] observed that general transcription factors Sp1 and CREB, as well as cell-specific factors AP2, Phox2a and Phox2b bind to proximal cis-acting elements and have a critical role in synergic activation of DBH gene trascription.
4) C-1021T is located in a noradrenergic cell type-specific DNAse I hypersensitive site of the DBH gene [25].

Two years later, Zabetian et al. [45] published their results of an experiment that investigated the LD structure of the DBH gene. They assumed C-1021T as a true functional polymorphism and examined the LD between C-1021T and another 11 markers, symetrically distributed around C-1021T, and what is the relationship of each marker to plasma activity. They have identified a block of LD at the DBH locus, including C-1021T that spanned across nearly 10 kb of its surroundings. All of these five markers within the LD block (-2124C->T, -1021C->T, IVS1+109G->C, 444A->G and IVS4+601C->T) are strongly associated with phenotype.

5´-ins/del This polymorphism, named 5´-ins/del, consist of 19 base-pair insertion/deletion approximately 4,7 kb 5´ from the transcriptional start site, -4784-4803del [11]. This region resides within the locus that Hoyle et al. [23] identified as a second positive regulatory element, between -1,5–5.8 kb (first between –600–1100 bp) that confers cell type-specific expression and contains an element responsible for the transient expression. 5´-ins/del is also associated with plasma DBH activity, particularly with deletion of lower and insertion of higher level of plasma DBH [11]. These results also showed that 5´-ins/del is in positive LD with another plasma DBH-associated polymorphism G444A and haplotype Del-A is associated with low plasma DBH activity in European American population.

TaqI The effect of this DBH SNP on the DBH levels is not completely understood. It is situated in intron 5 (IVS5+192C->T) and is easily genotyped by differential cleave with the restriction endonuclease TaqI [9].

ADHD, low DBH activity and genetic polymorphisms

In patients with the hyperkinetic syndrome and non-socialized conduct disorder, reduced DBH activity in serum and urine were recorded [5,42,43,37,38,18,20,6,7,]. Zabetian et al. [55] suggests, on the basis of their results and another hypothesis that low plasma DBH levels result from diminished expression of the DBH gene, that it is strongly associated with allele -1021T. Thus C-1021T, or another polymorphism in very tight LD with it, appears as a variant at DBH controlling plasma DBH levels and accounts for 35–52% of variation in plasma DBH activity. C1603T may explain additional 2% of variance. The low-activity 1603T allele is relatively rare, approximately 4% in European population [47]. SNP C1912T was not correlated to plasma activity and ADHD disorder. 5´-ins/del is also associated with plasma DBH activity, namely the deletion of lower and insertion of higher level of plasma DBH [11]. Wigg et al. [54] investigated the 5´-ins/del polymorphism (and another two: TaqI and $(CA)_n$ STR) in the group of 117 families with ADHD. They observed significant relationships between the genotypes of the three polymorphisms, but no biased transmission for either of the allele of the 5´-ins/del. They also found

no significant evidence for biased transmission of the haplotypes. Hawi et al. [21] observed the G444A polymorphism in connection with ADHD and found a slight increase in the transmission of allele 444A (allele 2), but it was not statistically significant. They also analysed markers creating a high-density map across and flanking of this gene and measured inter marker LD. Strong LD was detected between markers of polymorphisms G444A (exon 2) and TaqI (intron 5). Commings et al. [6,7] investigated whether TaqI B1/B2 may be associated with ADHD in a group of probands with Tourette's syndrome. They detected that Taq B1 allele (without TaqI site) was associated with the highest ADHD scores. Other research of this problem was done by Daly et al. [13]. They used a sample of ADHD children and found an association with TaqI (A1/A2) DBH allele A2 (present TaqI site). They both probably examined the same polymorphism, but with another nomenclature and another sample of probands, which may be the explanation for their different results.

Romain et al. [44] detected an association between DBH TaqI A2 allele and ADHD in a sample of 88 Brazilian nuclear families with ADHD, thereby confirming the previous report from Daly et al. [13]. The same results were also obtained by Inkster et al. [24] from their analysis of TaqI polymorphism in two independent samples of adults with ADHD and by Kirley et al. [29]. Wigg et al. [54] sought to replicate this work, but they found no significant evidence for the linkage of the TaqI A2 allele in the sample of 117 nuclear families with ADHD. Neither did Bhaduri et al. [3], who implemented the first molecular genetic study on ADHD in an Indian subject, exploring the transmission of G444A and TaqI polymorphisms in the DBH gene. On the other hand, Smith et al. [45] tested TaqI polymorphism in 105 Caucasian subjects with ADHD and ethnicity-matched controls. They observed that the DBH TaqI A1 allele was more frequently found in the ADHD group than the control group.

Discussion

Lower plasma DBH activity is caused by disappearing levels of circulation of the DBH protein, rather than decreasing the activity of enzyme. However, which polymorphisms play the main role in this process is not known yet. It could be the ones in the code region, or those in the regulation or non-code region. Hoyle et al. [23] suggested an essential domain between -600 bp and -1100 bp. Certain elements fundamental for human DBH gene are expressed in the noradrenergic neuron. Zabetian et al. [55] suggested low plasma DBH levels result from disappearing expression of the DBH gene strongly associated with allele -1021T. 5´-ins/del, located within the second positive regulatory element, may have additional effect on the expression. Allele 5´-del is associated with lower levels of plasma DBH [11]. The alteration of CSF levels of DBH protein and plasma DBH activity in coherence with polymorphisms localized in the code region is influenced by G444A with risk allele 444A, C1603T with relative rare risk allele 1603T (4% in European population) and G910T. Allele 910T (304S) codes the lower specific active form of protein. How does C1912T contribute to this is not exactly known. Although TaqI is localized in the non-code region, alterations in this area may have the decisive role on the final protein. Alterations may affect splicing due to origin or extinction of the artificial splice site, or some enhancer or silencer of splicing exists.

Only some of these polymorphisms were studied in connection with ADHD. Zabetian et al. [55] found that allele -1021T is associated with combined subtype of ADHD. Wigg et al. [54] observed a significant relationship of the genotypes of polymorphisms 5´-ins/del, TaqI and $(CA)_n$ STR in the families with ADHD, in the concrete TaqA2-del-A3 and TaqA2-ins-A4. Hawi et al. [21] found a slight increase in the transmission of allele 444A in the ADHD families. Association between TaqI and ADHD was also found by Comings et al. [6,7] (allele B1), Smith et al. [45] (allele A1), Daly et al. [13], Romain et al. [44], Inkster et al. [24] and Kirley et al. [29] (all with allele A2), but some results of other studies were negative [3,54]. This difference in the DBH TaqI A polymorphism could be attributed to population stratification, resulting in a false-positive association of the A1 allele with ADHD [45].

Which polymorphisms are the most important in the ADHD and low DBH plasma activity? Which of them have the major role and which of them cause additional effects? The role of other DBH polymorphisms is unknown because these polymorphisms were not studied in connection with ADHD. In patients with hyperkinetic syndrome and in non-socialized conduct disorder patients, reduced DBH activity in serum and urine were recorded [5,42,43,37,38,18,20,6,7].

Another question is the correlation between low DBH activity and prenatal hypoxia. Koudelová et al. [31] found that hypoxia (hyperbaric chamber) decreased the DBH activity in experimental animals (rats), particularly in very young ones (5 days after delivery).

Many conflicting suggestions may emerge as a result of diagnostic problems connected to ADHD with co morbidity and changes of symptoms in patients under 5 years of age or older than 10. Further investigation of polymorphisms in the DBG gene in connection with ADHD and DBH plasma activity should be done to provide a better understanding of this disorder.

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