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A GENETIC STUDY OF ATTENTION DEFICIT/
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LEEYOUNG PARK

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Karen Frederici
Major Professor's Signature

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A GENETIC STUDY OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER: CANDIDATE GENE ASSOCIATION STUDIES USING HAPLOTYPES

By

Leeyoung Park

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ABSTRACT

A GENETIC STUDY OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER: CANDIDATE GENE ASSOCIATION STUDIES USING HAPLOTYPES

By

Leeyoung Park

Attention deficit hyperactivity disorder (ADHD) is one of the most heritable complex disorders. Even with its high heritability, genome-wide scans do not show consistent results and candidate gene approaches have not been replicated in many cases. Such inconsistent results indicate the lack of a major gene effect, which reinforces the multigenic nature of ADHD, suggesting contributions from a large number of genes. In order to detect genetic contributions for mapping complex diseases, linkage disequilibrium (LD) has been the focus of recent research. Haplotype association studies use haplotypes that consist of several polymorphisms usually in linkage disequlibrium near the gene region, and consistently show better detection than single marker studies.

Through this thesis research, several important considerations in haplotype association studies were recognized. Two LD measurements, D' and r^2, differ depending on the relationship between polymorphisms, so it is critical to consider which combination of polymorphisms best captures the existence of risk alleles. Another consideration is that there may be several or more polymorphisms in a haplotype block that affect a phenotype in either a causative or a protective way. The third distinct point is that the detection power varies depending on the choice of association testing and the contribution of a polymorphism to the disorder.

Three candidate genes, the dopamine transporter gene (*SLC6A3*), the dopamine D_4

receptor gene (*DRD4*), and the α_2-noradrenergic receptor gene (*ADRA2A*), were selected depending on the catecholamine pathway, which is suspected to play a role in modulating the major psychopathology of ADHD. Recognizing the importance of phenotypes in association studies, gender difference and refined phenotypes were also studied. For gender difference, the data suggest that genetic susceptibility to ADHD is regulated differently in girls and boys. This posits important differences in the genetic susceptibility of the nervous system between genders, suggesting that the same polymorphism performs differently due to gender differences in dosage sensitivity in the catecholamine system.

This study reveals the association between all three candidate genes and ADHD supporting the catecholamine pathway as a main etiology. Through this research, possible major reasons for difficulties in mapping complex traits are identified. Moreover, by adding more clarification to the gender difference and phenotype of ADHD, this study provides a basic starting point for understanding the genetic etiology of ADHD.

To all (especially Jiehyun).

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KEY TO ABBREVIATIONS

ABI	Applied Biosystems Inc.
ADHD	attention deficit/ hyperactivity disorder
ADHD-C	ADHD-combined type
ADHD-PI	ADHD-predominantly inattentive type
ADRA2A	α-2A-noradrenergic receptor gene
ANOVA	analysis of variance
ASP	affected sib pair
bp	base pair
[^{123}I]β-CIT	[^{123}I] methyl 3β-(4-iodophenyl)tropane-2β-carboxylate
DA	dopamine
NE	noradrenergic
DAT1	dopamine transporter gene
DBH	dopamine β-hydroxylase gene
DISC-IV	the NIMH diagnostic interview Schedule for children, 4th edition
DRD2	dopamine D_2 receptor gene
DRD4	dopamine D_4 receptor gene
DRD5	dopamine D_5 receptor gene
DSM-IV	diagnostic and statistical manual of mental disorders, 4th edition
DZ twin	dizygotic twin
EEG	electroencephalogram
ETDT	extended transmission disequilibrium test
fMRI	functional magnetic resonance imaging

GABA	gamma aminobutyric acid
HRR	haplotype relative risk
LD	linkage disequilibrium
LOD	logarithm of odds ratio
MAP kinase	mitogen activated protein kinase
MLS	maximum LOD score
MZ twin	monozygotic twin
PCR	polymerase chain reaction
PKC	protein kinase C
QTDT	quantitative transmission disequilibrium test
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
SC6A3	sodium-dependent dopamine transporter
SLC6A3	dopamine transporter gene
SNP	single nucleotide polymorphism
SPECT	single photon emission computed tomography
TDT	transmission disequilibrium test
TSC	the SNP consortium
UTR	untranslated region
VNTR	variable number of tandem repeats

CHAPTER 1

Background

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a behavior disorder with strong heritability (0.7) characterized by marked and pervasive inattention, hyperactivity and impulsiveness resulting in impaired social and/or academic functioning[1]. It commonly affects 5-10% of children and adolescents and more than 3% of adults[2-6]. Boys are affected 3-8 times more frequently than girls[7]. ADHD usually occurs in conjunction with other major psychiatric disorders. Common comorbidity disorders and their relative frequencies are as follows: oppositional defiant disorder (33%), conduct disorder (25%), anxiety disorders (25%), depressive disorders (20%), and learning disabilities (22%). The comorbidity and recent genomewide scans suggest that ADHD is a polygenic disorder[8]. The studies for sibling relative risk and those on twins show significant genetic influence in ADHD[9-12].

Because ADHD is a common genetic disorder and patients' behavioral disabilities can affect not only the person and family, but also the society (school, workplace, etc.), the influence of ADHD is far-reaching. Also, through the inheritance of ADHD, similar problems are seen to continue through subsequent generations. For treatment of ADHD, various approaches, including medications, psychological remediations, and alternative treatments, have been employed[13]. Although several treatments have been successful in ameliorating ADHD symptoms, molecular-based remedies of ADHD depending on biological explanations are still in primitive stages of development.

With the increasing growth of high-throughput technology and bioinformatics, the genetic etiologies of many heritable diseases are unraveling one by one. However, like many other complex traits, even with high heritability, genetic study of ADHD is at an early stage. Different from typical family studies of single gene diseases in which linkages can be detected easily, finding quantitative trait loci for complex multigenic traits is very difficult even with very dense markers and larger families. Heritable psychiatric disorders like ADHD are considered one of the most interesting and important research areas due to possible revelations regarding the genetic background of brain function, yet there are many difficulties requiring not only profound genetic but also thorough phenotypic approaches.

Diagnosis and etiology of ADHD

The key characteristic of ADHD is a persistent pattern of inattention and/or hyperactivity-impulsivity, which are more frequent and severe than behaviors at a comparable developmental stage. In the case of mental retardation, an additional diagnosis is made for the child's mental age. Inattention is also observed in children with high intelligence when they are placed in academically understimulating environments. If symptoms are better explained by other mental disorders, ADHD is not diagnosed. Depending on the Diagnostic and Statistical Manual (DSM-IV), there are three subtypes of ADHD: the predominantly inattentive subtype, the predominantly hyperactive-impulsive subtype, and the combined subtype[14]. The diagnostic criteria for ADHD are summarized in Table 1.

Table 1. Diagnostic criteria for Attention-Deficit/Hyperactivity Disorder[14]

A. Either (1) or (2)

(1) six (or more) of the following symptoms of **inattention** have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level:

Inattention

(a) often fails to give close attention to details or makes careless mistakes in schoolwork, or other activities

(b) often has difficulty sustaining attention in tasks or play activities

(c) often does not seem to listen when spoken to directly

(d) often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (not due to oppositional behavior or failure to understand instructions)

(e) often has difficulty organizing tasks and activities

(f) often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (such as schoolwork or homework)

(g) often loses things necessary for tasks or activities (e.g., toys, school assignments, pencils, books, or tools)

(h) is often easily distracted by extraneous stimuli

(i) is often forgetful in daily activities

(2) six (or more) of the following symptoms of **hyperactivity-impulsivity** have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level:

Hyperactivity

(a) often fidgets with hands or feet or squirms in seat

(b) often leaves seat in classroom or in other situations in which remaining seated is expected

(c) often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings or restlessness)

(d) often has difficulty playing or engaging in leisure activities quietly

(e) is often "on the go" or often acts as if "driven by a motor"

(f) often talks excessively

Impulsivity

(g) often blurts out answers before questions have been completed

(h) often has difficulty awaiting turn

(i) often interrupts or intrudes on others (e.g., butts into conversations or games)

B. Some hyperactive-impulsive or inattentive symptoms that caused impairment were present before age 7 years.

C. Some impairment from the symptom is present in two or more settings (e.g., at school [or work] and at home).

D. There must be clear evidence of clinically significant impairment in social, academic, or occupational functioning.

E. The symptoms do not occur exclusively during the course of a Pervasive Developmental Disorder, Schizophrenia, or other Psychotic Disorder and are not better accounted for by another mental disorder (e.g., Mood Disorder, Anxiety Disorder, Dissociative Disorder, or a Personality Disorder).

There are strong genetic factors in the etiology of ADHD. Twin studies suggest high heritability ranging from 80-88%, and adoption studies using both adopted controls

and adopted cases also support the strong genetic component (47% of variance)[15]. Relative risk ratios of ADHD are $\lambda \cong$ 12-16 for MZ twins, $\lambda \cong$ 5-8 for DZ twins and first-degree relatives, and $\lambda \cong 2$ for second-degree relatives[9].

ADHD is a heritable disorder, but environmental factors underlie some causes of ADHD[16]. Those are traumatic brain injury and stroke, severe early deprivation, family psychosocial adversity, and maternal smoking during pregnancy.

Mode of inheritance and genome wide scans

The mode of inheritance is not clear in complex traits. Usually, polygenic or multifactorial transmission is suggested. A report describing a segregation analysis of ADHD rejects the multifactorial polygenic model using likelihood ratio tests[17]. However, it is clearly indicated by Morton, N. E., a developer of POINTER, which was used in the segregation analysis, "Conventional analysis of the mixed model concludes that a major locus is 'not proven', and so the most parsimonious polygenic model may well be correct."[18]. Because the likelihood does not differ much from each model and a false major locus model fits almost as well, the likelihood ratio tests may not be appropriate in this case and polygenic inheritance cannot be rejected in ADHD.

Genome-wide scans also support the polygenic nature of ADHD. The first genome scan using affected sib pair analysis of 126 pairs in 104 families resulted in no major gene with highest linkage peak of 2.6[19]. However, their follow up study of 277 affected sib pairs in 203 families found the first major susceptibility locus in a 12 cM region on chromosome 16p13 with maximum LOD score (MLS) 4.2, p-value = .000005[20]. This result suggests the possibility of major genes in ADHD, but more recent studies of the group support the polygenic property of ADHD. They found one more susceptibility

linkage on 17p11 from 270 affected sib pairs in 204 families using 10 cM markers[21]. In this report, the linkage signals were MLS of 2.98 for 17p11 and MLS of 3.73 for 16p13 (1 cM markers). With increased samples of 308 affected sib pairs in 226 families, the fine mapping (~2 cM) of nine susceptibility regions highlighted MLS of 2.55 for 5p13, MLS of 3.30 for 6q12, 3.73 for 16p13 (same as previous), and MLS of 3.63 for 17p11[22].

It is notable that the susceptibility regions from this group are completely different from the genome scans of other groups. A whole-genome scan (~10 cM markers) in 164 Dutch sib pairs suggests the linkages in 7p13 (MLS 3.04), 9q33.3 (MLS 2.05), and 15q15.1 (MLS 3.21) using narrow phenotypes[23]. Also, the genome scan using a population isolate in Columbia showed significant linkages on 4q13.2, 5q33.3, 11q22, and 17p11 in individual families[24]. Taken together, three genome scans suggest different loci for linkage of ADHD although 17p11 is common to two groups.

Depending on the results of current genome scans, there are at least 10 loci or more that contribute to ADHD. The important basic assumptions of these analyses are; 1) the alleles responsible for ADHD are identical by descent (IBD), 2) there are several major genes causing ADHD. The first assumption implies that the susceptible alleles are rare. Moreover, the series of genome scans support the locus heterogeneity of ADHD. If allelic heterogeneity is also true, then fine mapping narrowing down those regions may not be possible if not from a single family. The result of fine mapping of 16p13 is supportive for allelic heterogeneity because finer mapping results in less linkage signals.

These susceptible regions may be partially responsible for ADHD due to family-specific mutations in the regions. Or, those regions may harbor more causal genes together than other regions. As indicated previously, it is not known how many genes are involved or how they act together. Like other complex traits, it is only clear that a single

gene is not responsible for ADHD. Without any knowledge of the mode of inheritance, the conclusions about causal genes learned only from genome scans can be inappropriate.

Neurobiology of ADHD

The catecholamine system has long been suspected as a main rite of pathology of ADHD from neuropharmacology, neuroimaging, and animal models[25-27]. Studies in neuropharmacology were performed on stimulants to increase catecholamine neurotransmission as well as on non-stimulant[28]. The stimulant drugs are dextroamphetamine (d-amphetamine, Dexedrine), methylphenidate (Ritalin), pemoline (Cylert), and Adderall. Methylphenidate, the most common drug for the treatment of ADHD, primarily blocks dopamine reuptake but with some releasing effects. The effect of methylphenidate on norepinephrine is much lesser, and the effect on the serotonin system is minimal. Non-stimulants include the tricyclic antidepressants, the monoamine oxidase inhibitors, the aminoketone antidepressants, bupropion (Wellbutrin), the alpha-adrenergic agonists clonidine (Catapres) and guanfacine (Tenex).

More support for the catecholamine pathology can be found from animal experiments. Tyrosine hydroxylase gene inactivation results in dopamine deficient mice that are hypoactive[29], and knock out of the dopamine transporter gene showed high synaptic dopamine levels causing hyperactivity[30]. Moreover, animal studies revealed that selective lesions of the dopaminergic neurons cause significant alteration in attentional processes[31].

With neuroimaging studies on nigrostriatal and mesocortical distribution of dopaminergic neurons in the brain, cognitive impairments in ADHD were suggested due to a hypodopaminergic state in the prefrontal cortex and hyperdopaminergic state in

striatum[32,33]. One of the clear evidences for the dysfunction of fronto-striatal network in ADHD pathology comes from an fMRI study using response inhibition tests which are relevant for ADHD[34]. They tested response inhibition with and without drug for both cases and controls. Without drug, frontal activation was greater in ADHD children but striatal activation was less in ADHD children. However, with drugs, frontal activation was increased in both groups, but striatal activation was increased in ADHD and reduced in controls. Taken together, the prefrontal-striatal dysfunction is possibly due to hyperactive prefrontal region and hypoactive striatum through adrenergic and GABA system in striatum.

Candidate gene studies of ADHD

Due to the hypothetical pathology as described above, the catecholoamine pathway has been an important target for previous candidate gene approaches.[25,26,35] Both dopaminergic and noradrenergic systems are suspected to play roles in modulating the major psychopathology of ADHD. As Figure 1 shows, there are many candidate genes that may be responsible for ADHD in the catecholamine pathway.

Research on the dopamine model has focused on genes such as dopamine D_2 receptor gene (*DRD2*), dopamine transporter gene (*DAT1*, *SLC6A3*), dopamine D_4 receptor gene (*DRD4*), dopamine D_5 receptor gene (*DRD5*), and dopamine β-hydroxylase gene (*DBH*). Some of the research shows significant relationships between the specific alleles of the genes and ADHD, but many of these results could not be replicated in subsequent studies.[35] One of the well-studied genes is *SLC6A3*, the dopamine transporter gene. Most genetic association studies have been done using a variable number of tandem repeats (VNTR) in the 3' untranslated region (3'UTR). Many previous studies reported

that the most common allele, the 10 repeat allele, is associated with ADHD.[36-41] However, a considerable portion of these studies could not find an association between this VNTR of *SLC6A3* and ADHD.[38,42-45] Also, a meta analysis did not reveal significant association between 10 repeat allele and ADHD.[46] As with other examples of association studies between dopaminergic genes and ADHD, *DRD4* and *DRD5* showed some significant association although it was not always replicated. Although meta-analyses on *DRD4* and *DRD5* found reliable associations with ADHD, further detailed research is needed to clarify the inconsistency.[46,47]

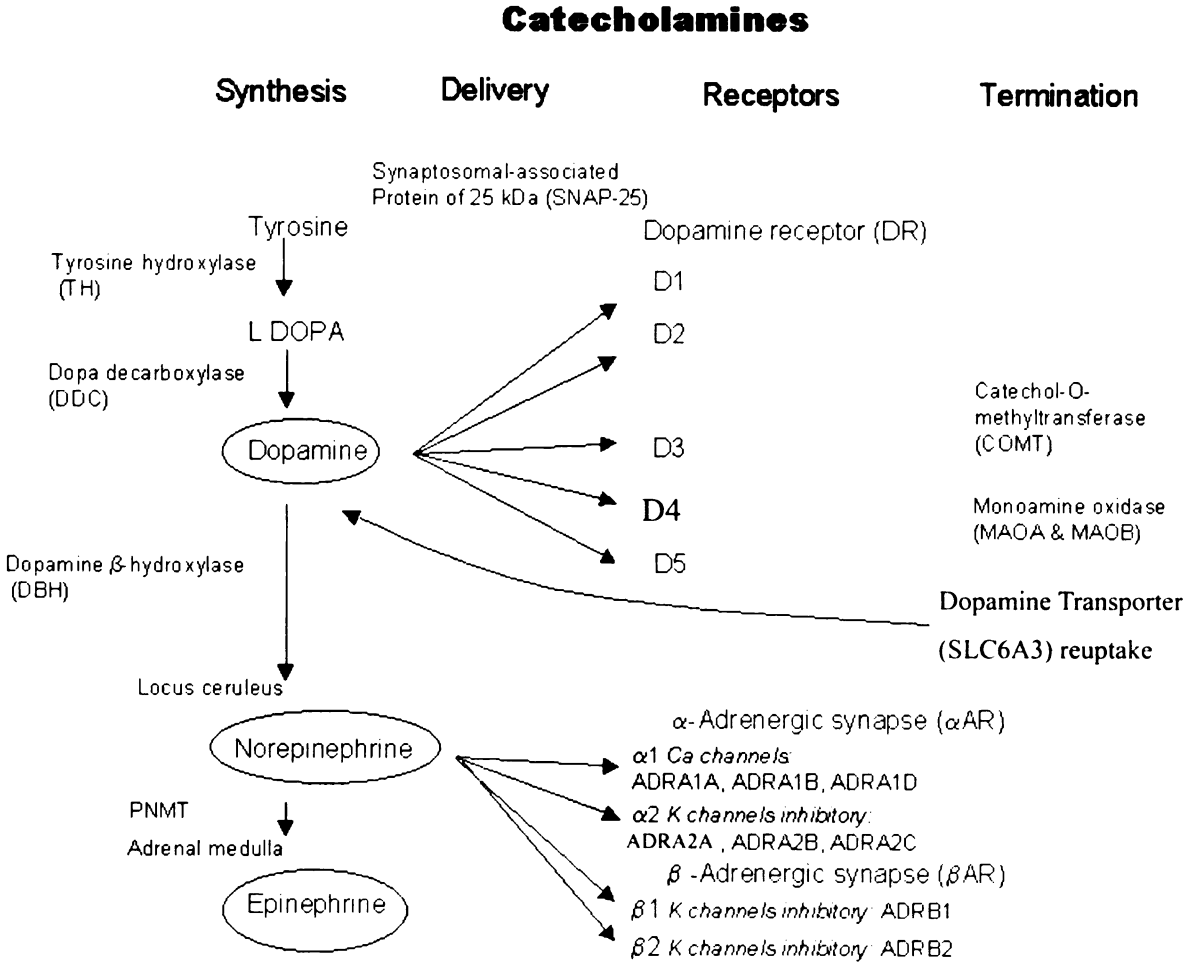

Figure 1. Catecholamine pathways and candidate genes.

The norepinephrine model is also highly favored based on animal models, pharmacological interventions, and the neural circuitry of attention processes. Among the genes involved in this process, the α_2-noradrenergic receptor gene is attractive particularly because clonidine, an α_2-noradrenergic receptor (*ADRA2A*) agonist, is a treatment drug for ADHD. The biological explanation for this is that the stimulation of presynaptic α_2-noradrenergic receptors results in inhibition of norepinephrine release into the synapse decreasing hyperactivity and increasing attention span. Association between one SNP on *ADRA2A* and ADHD had been examined and shown borderline significance, but could not be replicated consistently.[37,48-50]

Linkage disequilibrium and haplotype studies.

In order to detect minor genetic contributions for mapping complex diseases, linkage disequilibrium (LD) has been the focus of recent research. With high linkage disequilibrium, the polymorphisms that are closely linked together form regions that are called haplotype blocks. Within a haplotype block, several marker alleles in linkage disequilibrium with the risk allele are enough to map a complex trait. The usual measurement of LD is D' or r^2, that is significant generally if D' is higher than .7 and r^2 is higher than .3.[51,52] Large haplotype studies showed that the human genome of the world population consists of blocks of a few haplotypes with consistent recombinations.[53-55] However, a simulation study suggests genetic drift may generate block-like patterns of linkage disequilibrium.[56] Also, a linkage disequilibrium map of chromosome 22 revealed that many susceptible gene regions of schizophrenia did not show high LD.[57] These

results suggest that high density maps of disease loci are needed for mapping complex traits, such as ADHD.

Many haplotype studies of complex traits are ongoing and most employ a set of several haplotype-tag SNPs for testing association between haplotypes and diseases. Generally, haplotype approaches show better associations than approaches using single marker polymorphisms. As a target of methylphenidate, the stimulant drug for treatment of ADHD, *SLC6A3*, the dopamine transporter gene, is studied frequently.

SLC6A3, located in 5p15.33 telomeric region, spans 52,500 bps with 15 exons. The region from 10,000 bp upstream to 2,000 bp downstream contains a total of 337 SNPs, although most of them may be sequencing errors or rare mutations. A relatively high-density linkage disequilibrium map was constructed over this gene region and shows two clear blocks within the gene.[58] The second block beginning before exon 9 can be subdivided into two more blocks. Their haplotype association study on bipolar disorder revealed better association results than single marker or several markers located close to each other.[59] The first (5') haplotype block of *SLC6A3* did not show any significant association, but the second (3') block showed some significant association at a p-value < .05 level through the entire block with most of the SNP combinations. However, with most SNPs typed in the whole second LD block, the haplotype showed the most significant association by extended transmission disequilibrium test (ETDT). Haplotype association studies between ADHD and the *SLC6A3* gene have also shown better association results. A haplotype consisting of three polymorphisms, exon 9 SNP, intron 9 SNP, and the 3'UTR VNTR, was associated significantly with ADHD using the transmission disequilibrium test (TDT)[37]. Another haplotype study of a larger region of *SLC6A3* revealed a significantly biased transmission of a haplotype[60].

This better association using haplotypes was hypothesized to result from more sensitive detection due to the higher possibility of capturing the disease allele within a haplotype than a single marker. However, if the haplotype results were looked at more closely, sometimes the haplotype association studies showed significance even though a set of polymorphisms in low linkage disequilibrium was used.[60] Also, a set of polymorphisms that are in high linkage disequilibrium did not show a higher significance than using single marker allele.[59] It seems that some of the significant haplotype results might come from the combined effect of two or more different disease polymorphisms in different haplotype blocks. It should also be noted that the significance of association results could be strikingly different depending on which set of polymorphisms is chosen even in the same linkage disequilibrium block[58,59].

Some other studies suggested other possible effective polymorphisms.[61-63] It is reasonable to think that there are several polymorphisms in the *SLC6A3* gene locus that may act on expression, stability or other effects. A gene expression study using haplotypes of *SLC6A3* showed that promoter and intronic variants affect the transcriptional regulation of *SLC6A3* and suggested that particular combinations of polymorphisms in haplotypes affect the expression.[64] These results suggest that more careful approaches are needed in haplotype association studies considering not only the block size and LD but also the number of effective polymorphisms.

Phenotypic considerations.

With heritable complex traits of unknown etiology, the exact phenotype characterization is an important issue for genetic studies. Because there is no demonstration of consistent neurobiological differences in ADHD children, the

controversial phenotype definition and etiological heterogeneity may be the reason of invalidity in the genetic study. For ADHD, the fourth edition of diagnostic and statistical manual of mental disorders (DSM-IV) defines ADHD phenotypes as three subtypes, ADHD-combined type (ADHD-C), ADHD-predominantly inattentive type (ADHD-PI), and ADHD-predominantly hyperactive-impulsive type[14]. Research to date has been done on ADHD-C because it is the most prevalent. The ADHD-C and ADHD-PI are different cognitively and in familial history[65,66], although those are not differentiated consistently by the neuropsycological data[67,68]. These subtypes are coded on the basis of two different symptoms, inattention and hyperactivity-impulsivity that can be also considered possible separate phenotypes.

The consideration of phenotypes leads to the necessity of finding a consistent measurement for the genetic approach. One of the notable approaches is endophenotype. This concept came from the genetic theory of schizophrenia, having the synonymous meanings as "intermediate phenotype", "biological marker", and "subclinical phenotype"[69]. It can be defined as etiologically pure phenotype correlated with ADHD symptoms that is familial and appears in unaffected relatives.[70] The endophenotype should be associated with candidate genes and heritable. Because the biological endophenotypes are relatively more expensive to measure than congnitive endophenotypes, cognitive endophenotypes can be considered first.

Among several putative endophenotypes suggested, it is notable that the dysfunction of the response inhibition may be one of main etiologies in ADHD.[16,71,72] Disinhibition can be conceptualized as fast but inaccurate response, response perseveration, and a failure to respond appropriately in a response conflict task.[73] The possible endophenotypes of disinhibition in ADHD children are varied and were tested

for the possibility of a familial neuropsychological endophenotype.[71] Although those results show promising cognitive endophenotypes, it would explain the etiology of ADHD more precisely if the neurobiological function of the endophenotypes could be investigated in depth using neuroimaging or neurophysiological measurement.

One interesting feature of ADHD is the difference in prevalence between girls and boys. The ratio of boys to girls ranges from 3:1 to 8:1, and the ratio is higher in cases of clinically referred ADHD. Meta analysis on the gender difference in ADHD found that ADHD girls showed lower hyperactivity, fewer conduct disorders, lower externalizing behavior, and greater intellectual impairment (restricted to clinic-referred children), but there was no gender difference in impulsivity, academic performance, social functioning, and fine motor skills although most data were limited only to clinic-referred samples[7,74].

There are several hypotheses to explain the greater occurrence of boys with general childhood psychopathology. The most probable ones are the polygenic multiple threshold model and constitutional variability model. The former explains that girls need more genetic risk factors to be affected than boys and the latter describes that different casual factors affect females and males differently. The statistical test for the two models reveals an inclination to the polygenic multiple threshold model[75]. However, the difference in cognitive function between girls and boys suggests the possibility of the constitutional variability model. Moreover, recent research to find quantitative trait loci (QTL) related to cardiovascular functions using consomic rats showed that considerably different loci were related to cardiovascular function between women and men supporting the possibility of the constitutional variability model[76,77]. Study of genetic contributions to diseases which show gender specific predisposition may need to examine whether some risk alleles are gender specific.

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As a strong candidate gene of ADHD, the dopamine transporter varies across genders in respect to the expression and density of protein. In a rat study, the mRNA level and density of the dopamine transporter was significantly higher in females than males.[78] Combined with previous studies of the same group, it is suggested that such difference comes from a genomic effect of female gonadal steroids by comparison between ovariectomized females and intact males.[79,80] More interestingly, mRNA expression of the dopamine transporter is not regulated by estrogen in several brain regions including some striatum regions of female rats.[81] Ovariectomy in adult female rats reduces the dopamine transporter density but increases mRNA level, suggesting the involvement of other cellular mechanisms.[82] Also, in a human study, SPECT results show significantly higher density of the dopamine transporter in the striatal region of females.[83]

Gender difference in adrenergic receptors has been reported through cardiovascular studies.[84] Studies, that show antagonists for α_2-adrenoceptor affect male ejaculatory function, suggest further differences of the adrenergic system between genders.[85] Another study using an antagonist for α_2-adrenoceptor for tail artery of gonadectomy rats showed that gender differences in α_2-adrenoceptor function are not maintained by gonadal steroid hormones suggesting that the gender difference may be developmentally regulated.[86]

Although it has not been focused well, for association studies of candidate genes related to the catecholamine pathway, gender is a very important factor to consider in regards to ADHD.

Study Design

Although ADHD is highly heritable, genome-wide scans did not find a strong linkage nor any replicated regions that appeared interesting[20,23]. Inconsistent results are seen frequently in candidate gene approaches. The reason for the series of inconsistent results seems to come from the lack of a major gene effect reinforcing the multigenic nature of ADHD, which implies minor contributions from a large number of genes. Previous research indicates that the genetic study of ADHD requires more elaborate methods to determine genetic etiology.

Without clear pathology of ADHD, genome scans are attractive. However, currently, there is no good method for finding relevant genes in complex traits that are mutifactorial with heterogeneity. From the precept of previous genome scans of ADHD, candidate gene approaches were tried instead. For candidate genes, two drug target genes *ADRA2A* and *SLC6A3*, as well as *DRD4*, which has shown the most reliable association, were selected for candidate genes.

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Chapter 2

Candidate genes and initial analysis

Introduction

As described in the previous chapter, three candidate genes selected are relevant for ADHD, so these have been the focus of candidate gene studies of ADHD. *ADRA2A*, located in 10q24-26 in the middle of the chromosome, consists of one exon with a transcript of 3650 bp. *SLC6A3* is located in 5p15.3 near the end of the chromosome. This gene is quite large, approximately 52,640 bp consisting of fifteen exons. *DRD4* is 3398 bp also located in the telomeric region of chromosome 11 (11p15.5), and consists of four exons. In this chapter, the current information about the candidate genes is summarized first, and the analyzed data for the polymorphisms selected initially are described. Haplotype analysis was done on *ADRA2A* and is discussed in the later part of this chapter.

Candidate genes

α-2A adrenergic receptor gene (ADRA2A)

In molecular genetic approaches to ADHD, the most obvious target has been the catecholamine pathway, in part because it is the site of action of psychostimulants used to treat ADHD.[87] As a result, both dopaminergic (DA) and noradrenergic (NE) systems, which modulate one another, are thought to play roles in shaping the pathophysiology of ADHD. Both systems are expressed in the prefrontal cortex and its many projection regions. Accordingly, a number of prior studies have investigated DA genes with promising, but small, effects for *DRD4*, *DRD5* and *SLC6A3*[46,47,88].

In contrast, relatively little research has examined NE-relevant genes.

Noradrenergic neurons in the brain are concentrated in the brain-stem nucleus known as the locus coeruleus (LC). They project throughout the brain, providing the only source of noradrenergic stimulation to the prefrontal cortex and thus key NE-relevant genes are expressed in the prefrontal cortex and other brain regions relevant to the development of ADHD. Three types of noradrenergic receptors are traditionally recognized, alpha-1, alpha-2, and beta. Animal research suggests that NE projections in the prefrontal cortex enhance prefrontal cortical function primarily through post-synaptic alpha-2 receptors[89]. Of the several types of alpha-2 receptors in the brain, the most promising candidate for study is the α-2A adrenergic receptor (*ADRA2A*). This receptor is expressed in many areas of the brain, but is the most prevalent NE receptor type in the prefrontal cortex.

It is now relatively well established that NE is important to functions of the prefrontal cortex that are implicated as core deficits associated with ADHD, including working memory, focused attention, and response control[90]. As noted by Berridge[91], substantial data suggest that NE neurons are important in the regulation of arousal, wakefulness, and signal-to-noise ratio in attention. NE thus supports a key vigilance system in the brain[92]. The importance of NE to vigilance, alertness, and state regulation suggests its involvement in ADHD because difficulty with arousal and activation are core features of several theories of ADHD[93-95] and are noted as needing explanation in other theories[96]. As a result, dysfunction of the ascending NE system has often been theorized to mediate ADHD[97,98,99,100]. These theories are supported by substantial behavioral evidence suggesting that deficits in arousal and alertness are linked to ADHD. This evidence includes excess slow wave activity on EEGs[101], evidence of impaired signal detection using the d-prime parameter on Continuous Performance Tests[102], and slow and variable reaction times on fast reaction time tests in children with ADHD[94,103,104]. All of

these findings are consistent with abnormal functioning of a vigilance/arousal system that is likely mediated by ascending NE neurons, of which *ADRA2A* plays a key role in the prefrontal cortex.

Recent work implicates NE (as well as dopamine), and *ADRA2A* in particular, in tasks that reflect executive functioning in animals[89] and humans[105]. These functions, especially working memory, are involved in ADHD[96]. Pharmacological evidence in animals and humans also supports the role of NE, and in particular *ADRA2A*, in the prefrontal cortex and thus potentially in ADHD. The α-2A agonist clonidine has been used widely in the treatment of ADHD children[106], suggesting a potential role for the receptor in symptom expression. More definitive evidence emerges from recent work showing that the selective α-2A agonist guanfacine improves function on tasks reliant on prefrontal cortical functions in monkeys[90,107] and in humans[105], but does not affect behavior when the prefrontal cortex is not challenged[90]. Thus, pharmacologic investigations point to an important role for the NE system, especially the α-2A receptor, in the cognitive operations of the prefrontal cortex that are suspected of involvement in ADHD.

In short, there is ample evidence to suggest that NE neurons are important in ADHD and its associated multiple cognitive deficits[108], and at this initial stage of understanding an important NE receptor in the prefrontal cortex appears to be the α-2A receptor. It is therefore important to evaluate whether polymorphisms of the *ADRA2A* gene are related to ADHD in order to set the stage for further etiological studies.

Although the investigation of *ADRA2A* has only begun in relation to ADHD, association between ADHD or its symptoms and one SNP in the *ADRA2A* gene, rs1800544 (which creates an *Msp*I restriction fragment length polymorphism (RFLP)),

has been examined in four published studies. Comings, et al.[48] examined this association in children with Tourette's Syndrome, and found that the additive score of three noradrenergic genes correlated with expression of ADHD symptoms. A follow up report from this sample found that allele *m* of this SNP in *ADRA2A* was associated with ADHD symptoms[49]. However, Xu, et al. failed to find linkage and association with the same polymorphism using a transmission disequilibrium test (TDT) analysis in 94 nuclear families in which the proband had ADHD[109]. Roman, et al. studied 96 children with ADHD and their parents in a sample from Brazil. Although their haplotype relative risk (HRR) analysis with the disorder also yielded non-significant effects, this polymorphism was associated with ratings of inattention and hyperactivity, suggesting the possibility of an effect of the gene on symptom expression[50]. These two results both evaluated the G/G (alternatively denoted as *m/m*) genotype as the risk genotype. Nonetheless, it is difficult to draw clear conclusions about ADHD and *ADRA2A* from these few preliminary studies due to conflicting findings and the fact that a sample of Tourette's Syndrome patients provide the main positive findings, which may not generalize to other ADHD samples.

Dopamine transporter gene (SLC6A3)

One well-studied candidate gene on ADHD is *SLC6A3*, the dopamine transporter gene. Most genetic association studies have used a variable number of tandem repeat (VNTR) on the 3' untranslated region (3'UTR). Many previous studies reported that the most common allele, the 10 repeat allele, is associated with ADHD[36-41]. However, a considerable portion of these studies could not find an association between this VNTR of *SLC6A3* and ADHD[38,42-45]. Also, a meta-analysis did not reveal significant association between the 10 repeat allele and ADHD[46]. However, neuroimaging studies suggest the

involvement of the dopamine transporter (DAT) in the major etiology of ADHD[110]. A study using single photon emission computed tomography (SPECT) showed that ADHD patients (four women and two men) have an increase of 70% in dopamine transporter density over controls (total 30) with age-correction in striatum, suggesting the involvement of the dopamine transporter in the etiology of ADHD[111,112]. It is also known that the therapeutic treatment with methylphenidate reduces the increased DAT availability in ADHD adult patients[113].

The dopamine transporter is a member of a Na^+ and Cl^- –dependent transporter family, in forms of disulfide-linked homooligomer in membranes. It is known that the dopamine transporter interacts with the protein kinase C-alpha binding domain. There is direct evidence of phosphorylation and its regulation by PKC and MAP kinase, and it has several sites for N-linked glycosylation in the large second extracellular loop[114]. The dopamine transporter is located in the synaptic craft and highly expressed in the midbrain. Its main role is modulation of dopaminergic neurotransmission by the reuptake of released dopamine. It is supposed that some RNA editing occurs in the brain, and the dopamine transporter has several relevant protein sequences. Ensemble predicted three different mRNAs, but a study using rats could not find any alternative splicing in some brain regions[115]. The 12 transmembrane domains were well predicted from the multiple sequence alignment of the related transporters[116,117]. The strongly preferred transmembrane prediction suggests the 12 transmembrane domains starting from inside to outside of N to C terminal, and the positions of the transmembrane region in each sequence are identical (Figure 2).

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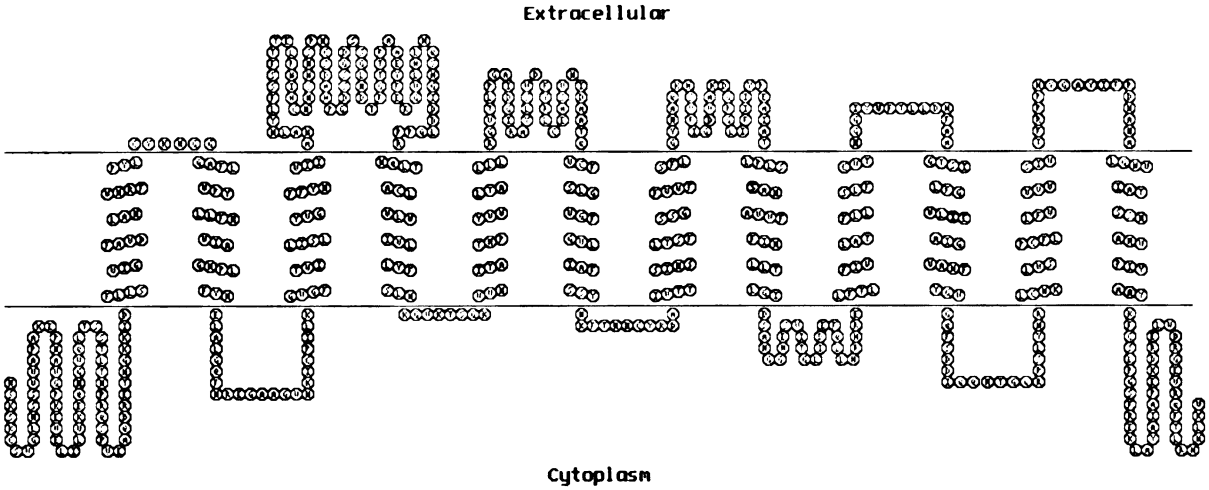

Figure 2. Schematic representation of dopamine transporter
(http://pharmacogenetics.ucsf.edu/set1/DAT/)

As described in chapter 1, the rather large *SLC6A3* gene region contains two LD blocks[58]. Depending on the literature, the second block is associated with bipolar disorder with p-value less than .05. Haplotypes consisting with three polymorphisms in the second block also result in significant association with ADHD through TDT. However, as mentioned in chapter 1, it is not clear if there is a functional polymorphism residing in the haplotype or the combined effect of several functional polymorphisms causes the association.

Dopamine receptor D4 gene (DRD4)

Dopamine receptor D4 is one of five subtypes of dopamine receptors, which belongs to the G-protein coupled receptor 1 family. The action of this protein is mediated by G proteins that inhibit adenylyl cyclase. Like other subtypes of dopamine receptors, dopamine receptor D4 contains seven putative transmembrane domains. However, this protein contains repeat variants that change the length of the protein in the putative

cytoplasmic part after the last putative membrane domain. In the genomic region, this variant is located in exon 3 and is the focus of association studies of psychiatric disorders.

Brain tissue examination showed that this gene is not imprinted in the human brain[118]. However, unlike the dopamine transporter gene that does not have any alternatively spliced isoforms in superior cervical sympathetic ganglia and dorsal root ganglia, alternative splicing transcripts for DRD4 were found in dorsal root ganglia[115]. There is no transcript of DRD4 in cervical sympathetic ganglia.

Dopamine receptor D4 gene is located in 11p15.5 near the telomere. There are four exons on the gene, spaced over 3398 bp. Most association studies have been done on two polymorphisms, 120 bp repeat promoter polymorphism and exon3 VNTR. As well summarized in a review article[119], the association results are inconsistent although meta-analysis showed the association of DRD4[46,47]. Interestingly, there is a report that, within VNTR subtypes, over 10 percent of ADHD probands have rare subtypes that were not discovered in the previous population studies[120]. Also, in Chinese Han population, ADHD children with normal IQ and methylphenidate responders showed the association of 2 repeat allele using ethnically matched controls[121]. These suggest that allelic heterogeneity of VNTR may contribute to the association and the subtypes may be different depending on the ethnicity.

TDTs using several more polymorphisms in the promoter region showed an association between –616 SNP and ADHD with p-value of .008, rather than no association of 120 bp insertion/deletion (I/D) polymorphism or VNTR[122]. There is no haplotype association study yet for *DRD4*. However, the LD structure of this gene region showed strong LD among the 7 repeat allele of VNTR for evidence of positive selection on this gene[123,124].

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Sample collection and demographic description

DNA samples were requested from affected children, their biological parents and one sibling nearest in age when possible. A total of 177 probands were studied in three groups: Non-ADHD Control (n=62), ADHD-C (n=81), and ADHD-PI (n=34). The majority of the probands were Caucasians (82%). The children were aged 7-13 (mean = 9.6), and included both boys and girls (64.5% boys). Complete trios were obtained for n=107 families. For buccal DNA preparation, a modified method described by Meulenbelt was performed in which cheek swabs were used for sampling followed by DNA preparation using phenol/chloroform purification (average 60 μg DNA per collection) [125].

A regular multistage recruitment and screening procedure was used to identify probands, based on the methods of the MTA studies. Families were recruited from the community using public advertisements and mailings to all parents of children in 2nd through 6th grades in the local school district. They were ruled out from participating if the index child had autistic disorder, bipolar disorder, Tourette's Syndrome, psychosis, history of head injury with loss of consciousness, history of seizures, or full scale IQ < 75 (evaluated with a 4-subtest short form of the Wechsler Intelligence Scale for Children, 3rd Edition).[126]

Index children were considered as *possible* ADHD if they either passed prescreen cut-offs on both parent and teacher versions of common ADHD rating instruments (Child Behavior Checklist or Teacher Report Form, Behavior Assessment Scale for Children Rating Scale,[127,128] or DSM-IV symptoms checklist)[129] *or* were previously diagnosed as ADHD (any type) by a physician or psychologist in the community who utilized teacher

and parent ratings to arrive at their diagnosis. Children were considered as possible controls if they were below cut offs on all of these parent and teacher scales and were never diagnosed with ADHD.

Final diagnosis was then determined after administration to the primary caregiver (usually the mother) of a structured diagnostic interview, the NIMH Diagnostic Interview Schedule (DISC-IV) for DSM-IV.[97] The DISC-IV is a widely used and accepted instrument with acceptable reliability and validity for evaluating diagnoses in community samples. Inter-rater reliability for ADHD diagnosis in our study was k=1.0, due to the computer-assisted nature of the interview procedure. After administration of the DISC-IV, an "or" algorithm was employed to identify ADHD.[130] If the child met onset, duration, and impairment criteria, had at least 4 symptoms on the DISC-IV, and exceeded the 90th percentile on teacher cut-offs, then a symptom was counted as present if it was endorsed on either the DISC-IV by the parent or by the teacher on the DSM-IV checklist ("sometimes" or "often" rated as "present"). In that way, a final symptom count was arrived at for each child and they were assigned to either the control group (4 or fewer symptoms) or one of the ADHD groups. Children with ADHD-hyperactive type were excluded as explained earlier. Also excluded were children with 5 symptoms of either inattention or hyperactivity, because their subtype status is indeterminate.[130]

Initial association results

For the preliminary association study of the dopamine transporter gene and dopamine receptor D4 gene, the most extensively investigated polymorphisms were chosen. For the non-stimulant medication system of ADHD on the noradrenergic system, *MspI* RFLP, which has been studied mostly on α-2A-adrenergic receptor gene, was

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chosen first. For this SNP, Comings et al.[48] examined children with Tourette's Syndrome, and found that the additive score of three noradrenergic genes was correlated with expression of ADHD symptoms. A follow up reported that allele *m* was associated with ADHD symptoms.[49] However, Xu et al. failed to find linkage with the same polymorphism using a TDT analysis in 94 nuclear families in which the proband had ADHD[109]. Roman et al. studied 96 children with ADHD and their parents in a sample from Brazil. Their HRR analysis also yielded non-significant effects, however the risk polymorphism was associated with ratings of inattention and hyperactivity, suggesting the possibility of a weak effect of the gene on symptom expression[50]. Those two results both demonstrated that the G/G (denoted as *m*/*m*) genotype is the risk genotype. In a case of small gene effect on ADHD, one SNP as a marker cannot show a significant association. So, all the publicly reported SNPs on the region were screened and two more SNPs were selected for this association study.

Table 2. Genotype Association Results (*p*-values of chi-square test).

Gene	Polymorphism	Control vs ADHD-C + ADHD-PI	Control vs ADHD-C
DRD4	Insertion/deletion	.01	.005
	VNTR	.90	.97
SLC6A3	Exon 9	.24	.24
	Intron 9	.07	.07
	VNTR	.05	.03
ADRA2A	rs1800544 (*Msp*I)	.77	.65
	rs1800545 (*Hha*I)	.83	.67
	rs553668 (*Dra*I)	.85	.77

Our genetic data revealed strong association with *DRD4* insertion (Table 2). The insertion/deletion polymorphism on the *DRD4* promoter region has been studied before for association with ADHD and the insertion allele is significantly associated with ADHD[131]. Our association result replicated this. We found that the *SLC6A3* VNTR 9 repeats associated with ADHD in this study samples (Table 2), however most association studies of the VNTR in *SLC6A3* showed 10 repeats as the risk allele. In our study, all alleles other than 10 repeats were significantly associated with ADHD. Another group that found the same result with this VNTR[45], and meta-analysis did not find a significant association between 10 repeats and ADHD[46]. This inconsistent result is not limited to ADHD. Other psychiatric or neuroscience research also found inconsistent association results for the VNTR of *SLC6A3*[132]. It can be hypothesized either that there is a real acting polymorphism which has a different pattern in linkage disequilibrium with VNTR or that the different VNTR subtypes make the difference working as an effective polymorphism.

Interestingly, the transmission disequilibrium test (TDT) did not show any significance between ADHD and either *DRD4* or *SLC6A3*. An association trial on parent groups found more significance between the parents of controls and the parents of probands. For the *DRD4* insertion/deletion polymorphism, the p-value was .007, and for *SLC6A3* VNTR polymorphism the p-value was .03. This means that the parent groups are already sorted significantly in the risk polymorphisms. With consideration of high heritability, it is reasonable that the parent group of probands has more risk genes because they may have expressed ADHD as children.

Despite the DSM-IV's identification of ADHD as a categorical disorder, many genetic analyses indicate that ADHD symptom dimensions have the same genetic

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influences at all levels of severity[133]. Those data commend consideration of the association of genetic markers with dimensional symptom ratings. We conducted such analyses using the quantitative transmission disequilibrium test (QTDT), with the advantage of considerably greater statistical power than is available in the conventional TDT analysis and inclusion of parent symptom counts. This approach revealed a trend toward significance using the MspI polymorphism (p=.022) and confirmed linkage between the T allele of *Dra*I and symptoms of inattention (p=.003). The T allele of *Dra*I is found primarily on a subset of chromosomes containing the G allele of the *Msp*I polymorphism. The results of QTDT suggest that the risk allele might reside on the chromosomes containing both the T allele of *Dra*I and the G allele of *Msp*I.

Table 3. TDT results for each ADRA2A SNP and ADHD subtype.

SNPs	ADHD type	T	NT	RR	χ^2	P value
*Msp*I (G allele)	ADHD-C	14	7	2.00	2.33	.13
	ADHD-PI	8	5	1.60	0.69	.41
	ADHD-(C+PI)	22	12	1.83	2.94	.086
*Hha*I (G allele)	ADHD-C	6	3	2.00	1.00	.32
	ADHD-PI	2	3	0.67	0.20	.65
	ADHD-(C+PI)	8	6	1.33	0.29	.59
*Dra*I (T allele)	ADHD-C	11	3	3.67	4.57	.033
	ADHD-PI	7	4	1.75	0.82	.37
	ADHD-(C+PI)	18	7	2.57	4.84	.028

ADHD-C: combined type. ADHD-PI: primarily inattentive. ADHD-(C+PI): both of the types. T: transmitted. NT: non-transmitted. RR: relative risk.

Using TDT, we found an association between ADHD and *Dra*I RFLP of the *ADRA2A* gene that did not show any significance in the case-control association study

(Table 3). The less common allele of the *Dra*I polymorphism was preferentially transmitted to ADHD children. It is interesting that this preferentially transmitted allele is less frequent in the athlete endurance group[134]. This result suggests that the *Dra*I RFLP may contribute only a minor portion to ADHD and could not be sorted in the case-control study, but instead preferential transmission was seen from the even distribution of parent groups.

Table 4. QTDT results for ADHD symptom dimensions

ADHD type	Marker	Allele*	X^2(df)	P
Inattention	*Msp*I	G	5.33(1)	.022
	*Hha*I	A	0.00(1)	NS
	*Dra*I	T	9.10(1)	.003
Hyperactivity-impulsivity	*Msp*I	G	4.85(1)	.037
	*Hha*I	A	0.05(1)	NS
	*Dra*I	T	6.95(1)	.015

Empirical p values are presented. NS: not significant.

*The allele conferring increased risk is denoted.

These results suggest that the differentiated effect between the major contribution and the minor contribution of genes should be considered. In case of the relatively major contributing polymorphisms, the case-control study might be useful, and, for detecting the minor contribution of polymorphisms, the transmission disequilibrium test would be helpful. The major and minor contributions can be different depending on the population sample, as an example, the clinic-referred children and the community-recruited children need to be considered differently. One more important point is that there needs to be a consideration of the moderate contribution of some polymorphisms that cannot be sorted

enough between parent groups and cannot be distributed evenly enough to be detected by TDT.

Initial haplotype analysis on *ADRA2A*

The variable results from previous association studies on *ADRA2A* may also be due to the difficulty of detecting minor contributions of a particular candidate gene to the liability for developing ADHD. This limitation is accentuated by restricting the analysis to the examination of a single polymorphism in the gene. If the selected polymorphism is, in fact, the only or primary functional polymorphism that contributes to the disorder being studied, it will then be the most robust marker for the disease. If it is not the functional polymorphism, however, then it would serve merely as a surrogate marker for the causative allele, and yield less robust findings in studies of association and linkage.

No evidence suggests that the rs1800544 SNP is functional. Therefore, to address this concern, we chose to examine more closely the haplotype structure of multiple markers in the *ADRA2A* gene and identify a set of SNPs to study. Previously, our case-control association studies on *ADRA2A* did not provide any significant results. Therefore, we used the transmission disequilibrium test (TDT) and quantitative transmission disequilibrium test (QTDT) to assess association and linkage of the *ADRA2A* gene polymorphisms with ADHD in two of its subtypes (ADHD-C and ADHD-PI) and in its two core symptom dimensions (inattentiveness and hyperactivity-impulsivity).

SNP selection

ADRA2A is a small gene with a genomic size of <4000 bp. The SNP Consortium (TSC) database identifies 12 variants, 8 of which are within or near the mRNA genomic

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region. A summary of the SNPs that have literature reports or frequency information is provided in Table 5. We include in Table 5 the frequency information from our own sample in the current study as well. The *Msp*I RFLP is located 5' of the transcribed region and the allele frequencies are similar in all Caucasian groups reported. There is a polymorphic SNP in the 5'-UTR, 3 non-synonymous mutations in the coding region, and two 3'UTR SNPs. As can be seen in Table 5, the allele frequencies observed in the study sample (labeled "Michigan" in the table) were typical of those reported in the literature.

We first examined the polymorphic status of the non-synonymous, coding SNPs, rs1800034, rs1800035 and rs180036, because variants at these positions have the potential to produce functional differences in the protein. We did not find these variants in our population, reinforcing the suspicion that these may be rare mutations. Three polymorphisms were chosen for analysis of the association with ADHD, based on their allele frequencies and their spacing in the genomic region. These were the *Msp*I RFLP (rs1800544) previously studied, a *Hha*I RFLP (rs1800545) in the 5' UTR, and a *Dra*I RFLP (rs553668) in the 3' UTR of the ADRA2A mRNA (Figure 3b).

As previously described, participants in 177 families were genotyped for the three SNPs in *ADRA2A*. For each of the markers, we evaluated Hardy-Weinberg equilibrium by simulation, using 10,000 iterations for each simulation. All of the markers appeared to be in Hardy-Weinberg equilibrium, as their one-tailed p-values were all non-significant (i.e., *Msp*I p = .355, *Dra*I p = .343, *Hha*I p =.719).

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Table 5. Polymorphism information for the *ADRA2A* locus.

SNPs	Frequencies		Population	Reference
rs1800544	C (M allele)	G (m allele)		
5' promoter region Msp I RFLP	.74	.26	Caucasian	[50] (Roman et al. 2003)
	.71	.29	Caucasian	[135] (Lario et al. 1997)
	.74	.26	Canadian	[109] (Xu et al. 2001)
	.73	.27	French, Irish & Scot	Canvas Database
	.67	.33	Mostly Caucasian	Michigan: Controls
rs1800545	G	A		
5' UTR	.89	.11	French & Irish	Canvas Database
HhaI RFLP	.88	.12	Mostly Caucasian	Michigan: Controls
rs1800034			Mutation	[136] (Feng et al. 1998)
rs1800035			Mutation	[136] (Feng et al. 1998)
rs1800038	C	A		
synonymou	.72	.28	Random	[136] (Feng et al. 1998)
s change	.71	.29	Japanese	JSNP Database
rs1800036			Mutation	[136] (Feng et al. 1998)
Dra I RFLP rs553668 3' UTR	C	T		
	.81	.19	Caucasian	[137](Hoehe et al. 1988)
	.80	.20	Caucasian	[134](Wolfarth et al. 2000)
	.81	.19	Mostly Caucasian	Michigan: Controls
rs3750625	C	A		
3' UTR	.73	.27	Japanese	JSNP Database

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Linkage disequilibrium and haplotypes

The linkage disequilibrium and haplotype were studied on three SNPs of the *ADRA2A* region. Linkage disequilibrium (LD) was calculated using D' and r^2 for each pairwise combination of SNPs (as shown in Figure 1a). Calculated from the GOLD software package[138], both D' and r^2 values show significant linkage disequilibrium between *Msp*I RFLP and *Dra*I RFLP, and it is likely that the *Msp*I RFLP shows a significant association in QTDT because this site is in linkage disequilibrium with the *Dra*I RFLP (Figure 3a). The D' value between the *Hha*I RFLP and *Dra*I RFLP is highest, while r^2 is very low. On the other hand, although the D' value between the *Hha*I RFLP and the *Dra*I RFLP was high, the r^2 was very low. This is thought to occur when the rare allele at one locus is linked to the common allele at the other locus and vice versa, rather than linkage occurring between alleles of similar frequency. [139]

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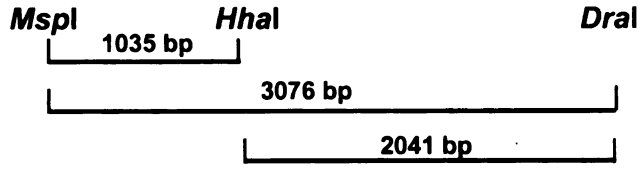
a. Marker linkage



	D'	r^2
	.827	.196
	.793	.351
	.832	.018

b. Genomic structure

C/G G/A C/T

c. Observed Haplotypes

			Frequency	Haplotype
C	G	C	69.1%	111
G	G	T	18.2%	212
G	A	C	10.7%	221
G	G	C	1.4%	212
C	G	T	0.6%	112

Figure 3. SNP location, linkage disequilibrium and haplotype distribution.

Panel **a** shows the distance and pairwise linkage disequilibrium between the SNP markers. Panel **b** shows the genomic structure of the ADRA2A gene. The transcribed mRNA is shown as a thick line and the portion that codes for protein is shown as a rectange. Panel **c** provides the nucleotide composition at each SNP for the observed haplotypes and frequency of each haplotype. Haplotype frequencies were determined using the EM algorithm.

Transmission disequilibrium testing using individual SNPs

The transmission disequilibrium test (TDT) was conducted to assess association and linkage with each of the 3 SNPs using the parent-offspring trio data described above. As shown in Table 2, the TDT revealed a significant association of the *Dra*I RFLP with the ADHD Combined subtype (p=.033). If both combined and primarily inattentive subtypes are considered together, a p-value of .028 was found. The less common allele of the *Dra*I polymorphism was preferentially transmitted to ADHD children. There was no preferential transmission of an allele of *HhaI* but transmission of the G allele of the *MspI* RFLP approached significance in the ADHD-(C+PI) group

The composition of our total sample, which contains non-disordered control children, some with intermediate symptom counts, allowed us to use tests such as the QTDT to assess association of each of the ADRA2A SNPs with the quantitative ADHD symptom dimensions (in addition to the diagnostic categories). We also had similar ADHD symptom data on parents. We included all symptom data (i.e., from case and control children as well as parents) in the QTDT analyses in order to make the symptom distribution resemble the population distribution as closely as possible. As shown in Table 3, both the inattentive and hyperactive-impulsive symptom scores showed association with the *Msp*I RFLP and even stronger association with the *Dra*I polymorphism. In contrast, neither symptom dimension was associated with the *Hha*I SNP. When results were repeated excluding parental data, these associations were similar but fell shy of significance.

Haplotype analysis

The finding of stronger association of ADHD subtypes and symptom dimensions with the *DraI* polymorphism than with the *MspI* RFLP suggests that the former may either be closer to a functional polymorphism or may "tag" a haplotype that contains the functional polymorphism. Therefore, the relations are determined among the alleles of each of the three SNPs tested in this study.

Table 6. Transmission disequilibrium test results for each haplotype and ADHD subtype.

Haplotypes*	ADHD type	T	NT	RR	χ^2	P value
111	ADHD-C	6	12	.50	2.00	.16
	ADHD-PI	3	7	.43	1.60	.21
	ADHD-(C+PI)	9	19	.47	3.57	.059
221	ADHD-C	3	5	.60	.50	.48
	ADHD-PI	3	2	1.50	.20	.65
	ADHD-(C+PI)	6	7	.86	.08	.78
212	ADHD-C	11	2	5.50	6.23	.013
	ADHD-PI	6	3	2.00	1.00	.32
	ADHD-(C+PI)	17	5	3.40	6.55	.011

ADHD-C: Combined subtype. ADHD-PI: Primarily Inattentive subtype. ADHD-(C+PI): both of the subtypes. T: transmitted. NT: non-transmitted. RR: relative risk. *For haplotypes: At each position 1= common allele, 2 = less common allele. For example: 111; common allele at *Msp*I, *Hha*I, and *Dra*I restriction sites, 212; rare allele at *Msp*I, common allele at *Hha*I, and rare allele at *Dra*I restriction sites. Empirical p values are presented.

In order to capitalize on the LD among the three SNPs in *ADRA2A*, we next conducted TDT analyses using multi-marker haplotypes to determine whether this yielded stronger results than tests performed with each SNP alone. These results are summarized in Table 6. The haplotype containing the rarer alleles of the *Dra*I and *Msp*I

RFLPs, and the common allele of the *HhaI* RFLP (i.e., haplotype 212 in Figure 3c) was preferentially transmitted to children with both subtypes of ADHD. The preferential transmission of haplotype 212 to affected offspring likely drives the marginal significance of haplotype 111, which is less frequently transmitted to affected children. Similar results (summarized in Table 7) were obtained using QTDT analyses of linkage and association between the ADHD symptom dimensions and the ADRA2A haplotypes for the entire sample, including both case and control children and parents.

Table 7. Haplotype analysis for *ADRA2A* using QTDT and ADHD symptom dimensions.

ADHD type	Haplotypes	X^2(df)	p-value	Direction
Inattention	111	6.00(1)	.016	Decreased risk
	211	NT	-	-
	221	0.03(1)	NS	-
	212	10.59(1)	.001	Increased risk
	112	NT	-	-
Hyperactivity-impulsivity	111	4.76(1)	.030	Decreased risk
	211	NT	-	-
	221	0.06(1)	NS	-
	212	10.55(1)	.004	Increased risk
	112	NT	-	-

At each position 1: common allele, 2: less common allele. For example: 111; common allele at *Msp*I, *Hha*I, and *Dra*I restriction sites, 212; rare allele at *Msp*I, common allele at *HhaI*, and rare allele at *Dra*I restriction sites. NT: Not Tested because of small number. NS: Not significant. Empirical p values are presented.

Discussion of initial haplotype analysis

The ADRA2A gene may be an important risk factor for ADHD in light of the role of its gene product in attention and the executive functions subserved by the prefrontal cortex and associated circuits thought to be involved in the disorder.[90] Despite its potential relevance, only a handful of studies have investigated the *ADRA2A* gene as a potential risk factor for the development of ADHD[37,48-50,140]. These studies analyzed an *Msp*I polymorphism in the promoter of the gene and looked for association with ADHD and/or its symptoms using a variety of statistical approaches. Comings, et al. found that in Tourette's Syndrome patients who also met DSM-IV criteria for ADHD, there was a modest correlation between symptom scores and the *Msp*I polymorphism, but the degree to which that sample represented the complete spectrum of ADHD patients is unclear. There are only two studies of ADHD children without Tourette's Syndrome, and these yielded incommensurate results. All of these previous studies relied on a single biallelic SNP to test for association between ADHD and *ADRA2A*, and thus did not adequately sample the array of alleles in this gene. This may lead to Type II errors in assessing the relevance of the gene to the etiology of ADHD. The present report excluded patients with Tourette's Syndrome and utilized a strategy of testing multiple SNPs and examining haplotypes. Based on a survey of the literature, it is the first study to do so with this gene in relation to ADHD. The positive results reported here therefore provide important new evidence that the ADRA2A gene is involved in the etiology of ADHD, and further clarify that the SNP assessed in prior studies may not be the most important marker in the gene with respect to risk for ADHD.

Three polymorphic SNPs spanning a 3 kb genomic region were chosen for the study of the *ADRA2A* gene. These SNPs are in moderate LD and define one common

haplotype (frequency = .69), in addition to two moderately frequent haplotypes (frequency =.18 and .11), in our control population (Figure 3c).

Analyses of the data using the TDT produced significant findings of association and linkage for two of the three SNPs tested. Previous studies implicated the m allele (the rarer G allele) of the *Msp*I marker in the risk for ADHD[48-50,140]. A trend was found for association and linkage between the m allele of *Msp*I and ADHD using the TDT (p=.13 for ADHD-C and p=.086 for ADHD-C+PI). In contrast, TDT analysis of the *Dra*I RFLP yielded significant results for ADHD-C (p=.03) but not for ADHD-PI (p=.37), as well as for both subtypes combined (p=.028 for ADHD-C+PI).

Despite the identification of ADHD as a categorical disorder in DSM-IV, quantitative genetic analyses suggest that ADHD symptom dimensions show similar genetic influences at all levels of severity.[133] These findings warrant consideration of the association of candidate gene markers with dimensional symptom ratings. We conducted such analyses using the QTDT. [141,142] This approach revealed significant association and linkage of symptoms of inattention with the rare allele of the *MspI* polymorphism (p=.022) and confirmed association and linkage with the rare allele of *Dra*I (*p*=.003). Similar findings were also obtained with these alleles and the hyperactive-impulsive symptom dimension (p=.037 for *MspI* and p=.015 for *DraI*). The results of the QTDT analyses suggest that the functional risk-inducing allele might reside on chromosomes containing the rare alleles for both *Dra*I and *Msp*I.

The TDT was repeated in order to evaluate association and linkage between ADHD subtypes and symptom dimensions and specific *ADRA2A* gene haplotypes. The haplotype containing the rare alleles of both the *Dra*I and *Msp*I markers was significantly associated with ADHD and the combined subtype. The QTDT results suggested that the

same haplotype was associated with severity on both the inattentive and hyperactive-impulsive symptom dimensions. These results suggest that the rare allele of *Dra*I may be closely linked to a functional polymorphism in the *ADRA2A* gene.

Conclusion

This haplotype study emphasizes several points concerning the search for functional alleles of genes that contribute to the inheritance of complex disorders and diseases. It is clear that marker selection in the candidate gene should not be limited to a single polymorphism. If the *Hha*I polymorphism had been the only marker selected for analysis, no indication of a significant association between ADHD and the *ADRA2A* gene would have resulted. In general, it is only when a functional polymorphism is being tested that a single marker will yield the most significant results. When the functional polymorphisms are not known, as is almost always the case, it is prudent to identify several polymorphisms in the candidate gene and to test each for association, both singly and in combination using the haplotypes that they constitute. In the case of *ADRA2A*, we identified three common haplotypes in the gene, the analyses of which allowed us to better demonstrate association and linkage between the ADHD subtypes and symptom dimensions and *ADRA2A*.

It is important to be aware that the power of the TDT depends not only on effect size and the mode of inheritance, but also on the allele frequency of the SNP in the population. In the present study, given our sample size we had adequate power to detect the largest effects in the ranges seen in our data within a multiplicative model for *DraI* and *MspI*, but we had much lower power to detect these effects for *HhaI*, given both its smaller effects and its much greater allele frequency. Furthermore, power was

considerably greater for the haplotype analyses than for the analyses of each individual SNP, underscoring the value of incorporating multiple markers in studies of association with candidate genes.

There are several limitations of this study that should be noted. Aside from the relatively small sample, the most important of these is the reliance on parental symptoms in the QTDT analyses. These retrospective symptom ratings are vulnerable to multiple biases, even when obtained in a careful structured interview as in our study. Therefore the quantitative results should be viewed with some caution until replicated. Nonetheless, we note that in our sample population the QTDT analyses relied only on allelic transmissions from parents and used the parents' symptom scores solely for the purpose of estimating the population mean of the ADHD symptom dimensions.

In conclusion for initial haplotype analysis, our results suggest that *ADRA2A* is associated and linked with ADHD and that the functional polymorphism is closely linked to *Dra*I. Although ambiguous haplotypes were not included, the haplotype TDT and QTDT results suggest that the functional allele is likely to be a less frequent allele (~.20) and is present on the "212" haplotype, which represents the rare alleles of *Msp*I and *Dra*I and the common allele at *Hha*I. It is possible that there is more than one functional polymorphism within this haplotype that contributes to the gene's effects. These results underscore the potential importance of noradrenergic systems in the etiology of ADHD.

Materials and methods

DNA Preparation

For buccal DNA preparation, a modified method described by Meulenbelt was performed in which cheek swabs were used as samples followed by DNA preparation

using phenol/chloroform purification (average 60 μg DNA per collection) using the following procedure[125].

1. Swabs collected in 5 ml of an STE solution were centrifuged at 2000 rpm in 50 ml tubes for 5 minutes.

2. Swabs, briefly suspended by a pulse vortex are inverted and transferred into a new 50 ml tube, and centrifuged at 2000 rpm for 5 minutes.

3. Swabs were pulled out and discarded using a clean glove after each set.

4. The liquid briefly suspended by a pulse vortex is transferred to a labeled 15 ml tube containing 5 ml of phenol/chloroform (1:1).

5. The tubes were inverted gently 10 times, and allowed to settle for 5-10 minutes.

6. The tubes were centrifuged at 4000 rpm for 8-10 minutes.

7. The aqueous layer of the solution was transferred to a new 15 ml tube containing 5 ml of chloroform, and the tubes were inverted gently 10 times.

8. The tubes were centrifuged at 4000 rpm for 8-10 minutes.

9. The aqueous layer of the solution was transferred to a new 15 ml tube containing 5 ml of 2-propanol, and the tubes were inverted 50 times.

10. The tubes were centrifuged at 4000 rpm for 8-10 minutes.

11. To leave the pellet, the solution was poured off carefully into a beaker, and 2 ml of 70% ethanol was added to the pellet. (After this procedure, the tubes could be stored in a refrigerator for overnight.)

12. The tubes were centrifuged at 4000 rpm for 5 minutes.

13. Ethanol was poured off carefully so that the pellet did not slide out from the tube, and the tubes were inverted onto clean papers to allow the pellet 20-30 minutes of drying time.

14. 200 μl of DNA hydration solution was added to the pellets, and let the tubes were left

to sit overnight at room temperature.

15. To dissolve the pellet, the solution was suspended by pulse vortex, and transferred to screw-capped tubes for storage at –20 °C.

The concentrated stock was diluted (1/100 or 1/50), and the diluted solution is read by UV spectrophotometer at 260 and 280 nm for the measurement of rough DNA concentration and purity.

Genotyping

Eight polymorphisms were studied from the three candidate genes by PCR and restriction fragmentation.

For *DRD4*, two well-replicated polymorphisms in ADHD, insertion/deletion promoter polymorphism and variable number of tandem repeats (VNTR) in exon 3, were selected and assayed with minor modifications[131]. The *DRD4* 120-bp tandem repeat polymorphism was assayed in 20 μl reaction mixture containing 20 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase with the same primer sets (5'-GTTGTCTGTCTTTTCTCA TTGTTTCCATTG-3' and 5'-GAAGGAGCAGGCACCGTGAGC-3'). Amplification was conducted under the following conditions with a hot start; an initial denaturing step at 94 °C for 3 minutes followed by 35 cycles consisting of 30 seconds at 94 °C, 30 seconds at 61 °C, and 1 minute at 72 °C, and the final extension step for 5 minutes at 72 °C using ABI 9700. The VNTR was amplified in 25 μl reaction mixture containing 100 ng of genomic DNA, 200 μM dNTPs, 0.5 μM of each primer, 1X Q solution, 1X Q PCR buffer, and 0.625 units of Taq DNA polymerase using primer sets, 5'-CGTACTGTGCGGCCTCAACGA-3' and 5'-GACACAGCGCCT GCGTGATGT-3'. The DNA was amplified with a hot start procedure including an initial denaturing step of

30 seconds at 96 °C, followed by 40 cycles consisting of 30 seconds at 95 °C and 90 seconds at 68 °C, and the final extension step for 4 minutes at 72 °C. After the amplification, the DNA was detected in 1.5 % argarose gel for the 120-bp tandem repeat polymorphism and 1.2% argarose gel for VNTR stained with ethidium bromide.

Three polymorphisms for *SLC6A3,* exon 9 SNP, intron 9 SNP, and VNTR in exon 15, were selected and typed with minor modification as described previously[37]. VNTR was amplified using the primer sets, 5'-ACTCCTTGAAACCAGCTCAG-3' and 5'-TATTGATGTGGCACGCACCT-3' in the reaction mixture containing 20 ng of genomic DNA, 62.5 μM each dATP, dTTP, dCTP, 31.25 μM dGTP, 31.25 μM deaza dGTP, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase using deaza dGTP as described[109]. The procedure includes an initial denaturation for 3 minutes at 95 °C, is followed by 35 cycles consisting of 30 seconds at 95 °C, 30 seconds at 58 °C, and 45 seconds at 72 °C, and the final extension step for 2 minutes at 72 °C. For amplification of the other two SNPs, the PCR used 60 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase (primer sets: 5'-CACAGCGTGGGCTCTGTG-3' and 5'-GGTGGAAGGAACCCAACTG-3' for the exon 9 SNP *and* 5'-GTCGTGCCGCCAT AGAAG-3' and 5'-CTGCACACAGAGGACAGGGT-3' which is mutated from the original sequence in the genome for a proper restriction cut for the intron 9 SNP). The cycling parameters involve an initial denaturation for 4 minutes at 94 °C; 35 cycles consisting of 40 seconds at 94 °C, 40 seconds at 65 °C for exon 9 SNP *and* 57 °C for intron 9 SNP, and 30 seconds at 72 °C; and the final extension step of 5 minutes at 72 °C. The amplified DNAs are digested by 10 units of restriction enzymes at 37 °C overnight (*Dde*I for exon 9 and *PflF*I for intron 9). For the efficiency reason, 1ul of

buffer 4 was added to the restriction digestion of the amplicon of intron 9. The DNA was detected in 1.5 % argarose gel for the VNTR polymorphism and 3% argarose gel for two other SNPs stained with ethidium bromide.

Three SNPs in *ADRA2A* were selected based on their spacing and frequencies as described previously[143]. The promoter SNP, rs180044 (*Msp*I RFLP), was typed by a modified amplification of the region using deaza dGTP as described[109]. Briefly, polymerase chain reaction (PCR) was performed in 20 μl reaction mixture containing 40 ng of genomic DNA, 62.5 μM each dATP, dTTP, dCTP, 31.25 μM dGTP, 31.25 μM deaza dGTP, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase. For the 5' UTR SNP, rs180045 (*Hha*I RFLP), PCR amplification were performed using primer sets, 5'-CCAAGTTATCAGGCCACCGA-3' and 5'-TGCTCCTGGCGGAACAT GAA-3' in 20 μl volume containing 40 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, 2 μl DMSO, and 0.5 units of Taq DNA polymerase. Amplification included an initial denaturing step at 94 °C for 3 minutes followed by 35 cycles consisting of 30 seconds at 94 °C, 30 seconds at 60 °C, and 45 seconds at 72 °C, and the final extension step of 5 minutes at 72 °C. After amplification, 10 units of *Hha*I restriction enzyme were added and digestion was performed at 37 °C for 2 hours. The region for the 3'UTR SNP, rs583668 (*Dra*I RFLP), was amplified in 20 μl volumes containing 40 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase (primer sets: 5'-TACAAGGGCATGGCTCACAA-3' and 5'-CCAAGGCCAGGATTTCAACA-3') using the same cycling parameters as above. Digestion of the PCR product was performed with 10 units of *Dra*I restriction enzyme at

37 °C for 2 hours. All restriction fragments were detected using 3% agarose gel stained with ethidium bromide.

Data Analysis

Hardy-Weinberg Equilibrium tests were performed using contingency tables. Case-control association was tested for each SNP. An increased alpha-level was considered using primarily the level of p= .01 to establish statistical significance in view of the number of statistical tests conducted to reduce the familywise Type I error rate, while preserving sufficient power to avoid excess Type II error. All statistical tests were two-tailed.

For within-family analyses of association and linkage between each of the *ADRA2A* SNPs and the ADHD diagnostic subtypes, we used the original TDT (i.e., a McNemar's chi-square test of biased transmission of alleles from heterozygous parents to their affected offspring)[144]. The quantitative TDT was performed using QTDT software[141,142]. Because of the very different distributions in ADHD symptom dimension scores between parents and their offspring, the polygenic variance (σ_g^2) as well as the additive genetic variance (σ_a^2) in the QTDT could not be calculated. This resulted in *p*-values that were very similar to empirical *p*-values calculated from 1,000 permutations.

One non-mendelian family (probably due to sample mix of the family) was not included in data analysis. In case of a strong suspicion of non-parternity of second child, the second child's genotype data was eliminated from the analysis. However, if the non-parternity corresponds to the first child who is phenotyped, the father's genotype data was deleted from the analysis.

Parents of controls were used to determine population haplotype frequencies. Both the use of manual procedures and the expectation maximization (EM) algorithm via

maximum likelihood estimation produced the same results for haplotype estimation. For analyses using haplotypes, where phase was ambiguous, the trios were omitted from the analysis (i.e., in 13 of 177 family samples). Linkage disequilibrium among the SNPs in *ADRA2A* was estimated using the GOLD software package[138]. We report findings separately for ADHD-C and ADHD-PI, as well as pooled results for both subtypes, in view of disagreement in the field about the degree of their etiological similarity and whether or not their results should be pooled[145].

Chapter 3

Phenotypic Consideration

Introduction

Unlike single gene disorders, complex phenotype presents much more difficulties in studying complex traits. Most complex traits include some degree of comorbidity and subtypes of the disorders. Although each disorder has a main pathophysiology, it seems to overlap at least partially with other similar disorders and the resulting symptoms can be somewhat distinguished depending on the clinical presentation. Complex traits can be frequently found in mental disorders due to the complex network of the brain.

As expected, ADHD often occurs in conjunction with other major psychiatric disorders. Common comorbidity disorders and their relative frequencies are as follows: oppositional defiant disorder (33%), conduct disorder (25%), anxiety disorders (25%), depressive disorders (20%), and learning disabilities (22%)[8]. The comorbidity of those disorders suggests an overlapping pathophysiology and possible genetic etiology with ADHD. It is not clear that the overlapping pathophysiology does mean stronger genetic influence of the comorbid genetic locus. It is worthwhile to examine the comorbidity and distinguish the etiology, but, in the current stage of the genetic association studies, to find out each genetic etiology of the disorder seems more appropriate.

In this chapter, to find out if a polymorphism is associated with ADHD phenotype, DSM-IV based case-control and TDT test are primarily considered including their subtypes. A total of 228 nuclear families that is slightly more than were used in chapter two were studied in three groups: Non-ADHD control (n=70), ADHD-combined ype (n=95), and ADHD-primarily inattentive type (n=29) with 64.4% Caucasians and

67% boys. This sample population is a balanced collection between cases of controls of several ethnic groups (Caucasian, African American, Hispanic, Asian, American Indian, and mixed others) with p-value of .57, so that the case-control association is not affected by population stratification. The final sample included children aged 6-13 years (mean = 9.6). To address concerns in regard to ADHD phenotypes, several relevant endophenotypes were tested also. Finally, one important yet unexplained feature of ADHD, the gender difference, was addressed.

Similar to those of chapter two, the children were recruited via a community-based, multi-gate strategy in which more stringent diagnostic procedures were applied at each stage in order to establish cases. In the first stage, common rule outs were identified such as autistic disorder, mental retardation, neurological disease, and sensorimotor handicap. In the second stage, parent and teacher normative ratings were obtained to make sure the child had elevated levels of behavior problems in both settings (for potential ADHD participants) or had normal range behavior across settings (for potential control participants). During the final stage, a structured diagnostic interview (the NIMH Diagnostic Interview Schedule for DSM-IV) was performed with the primary caregiver to establish that full DSM-IV criteria were met for the ADHD groups and that Control children did not have ADHD. Parent and teacher data were combined in an "or" algorithm to arrive at the final symptom count in assigning the ADHD subtype. Thus, if a symptom was endorsed by the parent on the DISC-IV, or was rated by the teacher as a "2" or a "3" on the 0-3 scale used to rate the items ADHD Rating scale, it was counted as present. At this final stage, we also assessed other psychopathologies, ruling out children with Tourette Disorder, bipolar disorder, psychosis, or learning disability and recording other comorbid conditions for secondary data analysis.

DSM IV based associations.

As described previously, there are three subtypes of ADHD. Two relatively common subtypes, ADHD-C and ADHD-PI types, appear to differ both cognitively and with regard to familial history[65,66,146]. Although neuropsychological data has not differentiated them[147,148], there is a suggestion that the two subtypes are entirely different disorders[145]. Moreover, there is evidence that the comorbid symptoms with other disorders differ depending on the subtypes[149].

The concern, that the ADHD-combined type (ADHD-C) and ADHD-predominantly inattentive type (ADHD-PI) are totally different subtypes of ADHD, leads to consider segregated analysis between two subtypes. Although previously discussed that ADHD combined and inattentive subtypes may be distinct conditions[145], it is also noted that there is an argument that these two ADHD subtypes commonly share many neuropsychological features[150]. Also, the inattentive type may to a large extent represent a milder version of the ADHD combined type[151]. Therefore, the tests were conducted on both ADHD-C and ADHD-PI groups, as well as the combination of the two for purposes of the present paper to maximize power. TDTs and case-control tests were conducted on those groups.

In Table 8, ADHD-PI shows generally reduced association probably due to the smaller sample size. There is no major difference in the trend of associations between ADHD-C and ADHD-PI. There are some differences in association levels between these two groups, but it is hard to surmise further due to the small sample size. With the similar trend between ADHD-C and ADHD-PI, the combined grouping of both and ADHD-C also show similar results. More sampling on ADHD-PI seems necessary for further

speculation. These results indicate that, at least in our sample population, the genetic etiology of ADHD in three genes may be similar.

Table 8. Genotype Association Results (p-values of chi-square test).

Gene	Polymorphism	Control vs ADHD-C + ADHD-PI	Control vs ADHD-C	Control vs ADHD-PI
DRD4	Insertion/deletion	.003	.003	.20
	VNTR	.76	.99	.18
SLC6A3	Exon 9	.82	.67	.88
	Intron 9	.046	.06	.11
	VNTR	.25	.32	.32
ADRA2A	rs1800544 (*Msp*I)	.52	.40	.98
	rs1800545 (*Hha*I)	.43	.55	.52
	rs553668 (*Dra*I)	.099	.079	.58

Exact numbers for ADHD-C + ADHD-PI are indicated in Tables 13-15.

Although our sample population is balanced with respect to cases and controls in ethnic groups, the same case-control tests using only Caucasians were tested to address if there is ethnic-specific association. Overall, the results (Table 9) were not much different from the previous results in Table 8. Some changes in Caucasian only associations are summarized below; the association of insertion/deletion polymorphism in *DRD4* was reduced and the association of VNTR in *SLC6A3* was enhanced. One interesting feature is that the association of VNTR was strongest in the case-control test using only ADHD-PI although it is smaller sample size. It is noteworthy that, as described in the previous chapter, this association is in the opposite direction compared to the research of other groups. Further discussion regarding this question is addressed in Chapter 5. The

Caucasian-only association suggests that there may be some ethnic differences in the level of association of each polymorphism depending on the LD with functional polymorphisms, although further research using increased samples is necessary.

Table 9. Genotype Association Results (p-values of chi-square test on only Caucasians).

Gene	Polymorphism	Control vs ADHD-C + ADHD-PI	Control vs ADHD-C	Control vs ADHD-PI
DRD4	Insertion/deletion	.062	.13	.16
	VNTR	.31	.28	.56
SLC6A3	Exon 9	.85	.90	.71
	Intron 9	.054	.073	.11
	VNTR	.046	.09	.039
ADRA2A	rs1800544 (*Msp*I)	.84	.71	.79
	rs1800545 (*Hha*I)	.30	.11	.72
	rs553668 (*Dra*I)	.21	.039	.55

TDT shows a pattern similar to that described in the previous chapter with smaller samples (Table 10). None of polymorphisms in *DRD4* and *SLC6A3* is significantly associated with ADHD using TDT, and again the *Dra*I RFLP in *ADRA2A* shows borderline significance reduced a bit more than the result in the previous chapter. As noted in Table 8, slightly increased sample size produced more significant association of *Dra*I RFLP in *ADRA2A* using the case-control test. It appears that the detection of association is moved from TDT to case-control test in *ADRA2A* after adding a few more samples. This result is probably due to the fact that both the added ADHD subjects and their parents do have more risk alleles of *Dra*I. Overall difference patterns between subtypes are similar to the case-control association studies.

Table 10. Transmission disequilibrium test results for each *ADRA2A* SNP and ADHD subtype.

SNPs	ADHD type	T	NT	RR	χ^2	P value
	ADHD-C	17	9	1.89	2.46	.12
*Msp*I (G allele)	ADHD-PI	10	6	1.67	1.00	.32
	ADHD-(C+PI)	27	15	1.80	3.43	.06
	ADHD-C	5	7	.71	.33	.56
*Hha*I (G allele)	ADHD-PI	4	2	2.00	.67	.41
	ADHD-(C+PI)	9	9	1.00	0	1.00
	ADHD-C	14	5	2.80	4.57	.039
*Dra*I (T allele)	ADHD-PI	8	6	1.33	.82	.59
	ADHD-(C+PI)	22	11	2.00	4.84	.056

ADHD-C: combined type. ADHD-PI: primarily inattentive. ADHD-(C+PI): both of the types. T: transmitted. NT: non-transmitted. RR: relative risk.

Recognizing that ADHD is a problem of degree rather than existence and that this sample population contains many controls and some intermediates, the quantitative TDT measurement of ADHD is employed. There are nine symptoms of inattention or hyperactivity-impulsivity on DSM-IV criteria that defines ADHD if an individual has at least six symptoms. The number of symptoms is used for quantitative analysis for the association. Recognizing that σ^2_g and σ^2_a are not calculated using parents' and their children's symptoms in our sample population which is the same as the smaller samples of the previous chapter, the variance component tests were not permutated.

Same as TDT, the QTDT tests did not demonstrate any association with polymorphisms in *DRD4* or *SLC6A3*. However, as summarized in Table 11, *ADRA2A* shows an association with ADHD. Again, these associations are reduced compared to the

smaller samples in the previous chapter. Given the importance of quantitative measurements, the QTDT using symptom counting is robust in replacing TDT especially in our sample population, which has controls as well as subjects with intermediate symptoms.

Table 11. QTDT results for ADHD symptom dimensions associated with *ADRA2A* polymorphisms.

ADHD type	Marker	Allele*	X^2(df)	P
Inattention	*Msp*I	G	2.81(1)	.09
	*Hha*I	A	0.23(1)	NS
	*Dra*I	T	4.76(1)	.029
Hyperactivity-impulsivity	*Msp*I	G	2.24(1)	NS
	*Hha*I	A	0.62(1)	NS
	*Dra*I	T	4.05(1)	.044

NS: not significant.

*The allele conferring increased risk is denoted.

Endophenotypes

Attention deficit hyperactivity disorder (ADHD) is a common, costly[152], and impairing[150] condition typically diagnosed in childhood. It evidences substantial heritability[153] and as a result, molecular genetic studies have proceeded rapidly, with replicated findings for several candidate genes[154]. Recognizing the measurement of clear phenotype, the endophenotype, quantifiable and intermediate constructs, becomes the immediate interest in the genetic research of ADHD. Therefore, finding the measurable phenotypes is an important step in the genetic study[155]. The appropriate endophenotypes can be: 1) correlated with ADHD symptoms or disorder; 2) amendable to objective,

ideally quantitative, measurement; 3) present in relatives; and 4) theoretically or empirically related to the etiology of the disorder. The expectation is that endophenotypes involve the same biological pathways as the disease but are nearer the relevant gene action. In this study, the neurocognitive measures were focused on due to their theorized mediating causal role in ADHD.

Among the relevant neurocongitive circuits of ADHD, evidence from neuroimaging studies converges on the involvement of frontal-striatal-thalamic dysfunction in ADHD with involvement of the prefronatal cortex, basal ganglia, cerebellum and corpus collosum[33,156]. To date, the primary theoretical and empirical emphasis has relied on catecolamine transmission, partly because this is presumed to be the site of psychostimulant action used in treating ADHD.

Depending on the neuropsychological basis, possible neurocognitive endophenotypes can be found in executive functions and inhibitory controls. One such possible endophenotype of ADHD is a difficulty to suppress non-goal motor response or competing response tendencies. The frontal-subcortical-thalamic circuits are important in suppressing competing behavior as well as in representing intended behavior. Operational definitions of behavioral suppression have led to several candidate endophenotype measures, of which perhaps the most well established is the Logan stop task[157].

Motor inhibition or response suppression is operationalized with the tracking version of the stop task, a computerized choice-reaction time task using the same procedures as Logan et al.[157] and Nigg[158]. In the tracking version of this task, stop signal reaction time, the index of inhibitory control, is estimated by subtracting mean stop signal latency from mean go response time[157]. Go response time and variability of response time serve as indices of regulation of arousal, activation, or effort. Each of the outcome

variables is mean reaction times with excellent reliability.

In this study, the tracking version of the stop task was used, which is supported by recent data as providing the most robust assessment of stop signal reaction time (stop signal RT)[159]. Generally, ADHD children have deficits on the speed and variability measures. Both stop signal reaction time and response variability show the strongest correlations between probands and relatives among several candidate measurements. Recognizing the nature of those endophenotypes, the quantitative analysis was primarily tested using the QTDT software.

Table 12. Endophenotype analyses (p-values of tests for total evidence of association and QTDT).

Gene	Polymorphism	Stop Signal RT		Response variability	
		Association	TDT	Association	TDT
DRD4	Insertion/deletion	NS	NS	NS	NS
	VNTR	NS	NS	NS	NS
SLC6A3	Exon 9	NS	NS	NS	NS
	Intron 9	.0569	NS	NS	NS
	VNTR	NS	NS	NS	NS
ADRA2A	rs1800544 (*Msp*I)	NS	NS	NS	NS
	rs1800545 (*Hha*I)	.0157	NS	NS	NS
	rs553668 (*Dra*I)	NS	NS	NS	NS

NS; not significant with p-values higher than .10.

Tests of quantitative associations and transmission disequilibrium were summarized in Table 12. Because parent phenotypes are not included, all the variances are the same in those analyses. There is no significant population stratification except the

polymorphism on Exon 9 of *SLC6A3* on the response variability (χ^2 = 4.84, p-value = .0278). Unlike association results using DSM-IV based phenotypes, most of the tests fail to find associations between those endophenotypes and polymorphisms. One interesting result is the association between *Hha*I RFLP on *ADRA2A* and stop signal reaction time. Recognizing that the *Dra*I RFLP on *ADRA2A* is associated with DSM-IV based phenotypes, this endophenotype may be associated with the gene, *ADRA2A*, in different ways than the DSM-IV phenotypes.

Although candidate endophenotypes are familial and correlated with ADHD, a cognitive endophenotype may be partially overlapped with ADHD so that the children without ADHD also have the endophenotype. It is possible that a genetic study on the endophenotype can dilute the pure genetic effect on ADHD. Another concern on the endophenotypes is the endophenotype may contain other sets of genes affecting the endophenotype. As an example, the stop signal task may include the genes related to the peripheral nervous system, although ADHD mostly concerns the central nervous system. This result suggests that the endophenotypes may need to be cautiously selected for the genetic association study only if they are proven to be heritable and to be exact subsets of ADHD or biologically relevant to ADHD depending on neuroimaging or neurophysiology.

Gender difference

Under-examined in the genetic literature, like most other psychopathology, ADHD does not afflict boys and girls equally. In childhood, the ratio of affected boys to girls ranges from 3:1 to 8:1, depending on whether one surveys community or clinical samples[160]. It has been unclear whether this difference in prevalence reflects merely

differences in socialization or diagnostic detection of boys versus girls[161] or whether girls are protected from the risk factors that cause the disorder in boys[75,162]. Supporting this latter perspective have been meta-analytic findings that although girls with ADHD tend to have less severe behavioral disturbance than boys in terms of less severe hyperactivity and conduct problems, they may have greater intellectual impairment[7,74]. One neuroimaging study found more marked hypoactivation of brain region(s) in girls than boys with ADHD[163], although results need replication. Nigg et al. found that girls with the ADHD inattentive type had deficits in response inhibition that were not observed in boys with the inattentive type of ADHD[164]. Numerous neurodevelopmental and behavioral differences between boys and girls, rooted in their obvious differential hormonal exposures during prenatal development and subsequently[165,166], can be noted to support the possibility of distinct neurobiological mechanisms underlying ADHD in boys versus girls.

This possibility has already been noted in other areas of psychopathology. For example, protective effects of estrogen may account for delayed onset and reduced severity of schizophrenia, whereas estrogen withdrawal may be implicated in an increased incidence of Alzheimer's disease in post menopausal women[167,168]. However, unlike schizophrenia or Alzheimer's, ADHD begins in early childhood, before significant sex differences in circulating estrogen arise. Although the disorder can persist into adulthood, the gender ratio in adults is unclear[169]. Thus, hormonal effects at puberty could still affect ADHD prevalence later in development. Even so, childhood differences in expression of ADHD may be attributable to prenatal hormonal effects on neural organization[170].

With regard to candidate neural systems and genes, ADHD is widely suspected

of involving catecholaminergic dysfunction[25,26]. Both dopaminergic and noradrenergic systems are thought to be important. Gender differences in regulation of these systems would not be unprecedented; for example, gender differences have been noted in relation to cardiovascular disease both in the adrenergic system[84,171,172] and in animal studies of genetic correlates looking at quantitative trait loci[76,77]. One noradrenergic candidate gene for ADHD, the α-2A-adrenergic receptor (*ADRA2A*), apparently shows gender specific differences in response in relation to vasoconstriction levels in animals[86].

Also suggestive is that gender differences have been noted in physiological response to cocaine and methamphetamine, which are dopaminergic agonists closely related to methylphenidate, a common treatment for ADHD[173,174]. Striatal dopamine concentrations of methamphetamine treated female mice were significantly less depleted than those of identically treated male mice. Evidence suggests that women may have a higher synaptic concentration of dopamine in the striatum than men[175]. Furthermore, fluctuating ovarian hormones cause periodic variation in the expression of dopamine receptors in females[81,176]. Moreover, the mRNA level and density of the dopamine transporter are significantly higher in female rats than in males[78]. In human studies, SPECT indicates a significantly higher density of the dopamine transporter in the striatal region of females than of males[83], and such effects have been hypothesized as potential mechanisms in ADHD's gender differences[177]. Animal studies suggest that such differences may be related to female gonadal steroids regulating gene expression[79,80]. Therefore, we sought to evaluate whether the three candidate genes selected in this study show differential associations to risk in boys versus girls.

DRD4.

In Table 13, the association between polymorphisms in the *DRD4* gene and

ADHD is examined. In the total sample, individuals, who are homozygous for the insertion in the *DRD4* promoter, were at an increased risk for ADHD. This was a very significant finding in boys but not significant in girls, although girls did show a similar trend. No significant association with the *DRD4* VNTR was found in our sample population.

When we excluded non-Caucasians from the data set and recalculated the association between *DRD4* I/D and ADHD, the trends were very similar but, probably due to the reduced sample size, did not reach greater significance (boys: p= .02; girls: p= .42; overall: p=.06). No significant difference between boy and girl controls or cases was found (control: p = .15; cases: p=.46).

SLC6A3.

Three polymorphisms were similarly studied for *SLC6A3* (Table 14). The haplotypes using these polymorphisms were previously associated with ADHD[37]. No significant associations were found for the exon 9 SNP. For the intron 9 SNP in *SLC6A3*, a trend was found in the total cases vs. controls for increased risk in the individuals carrying the G allele. When the genders were considered separately, girls had a very significant ADHD risk associated with the G allele; boys did not. When only Caucasians were tested, similar results were obtained (boys: p= .74; girls: p= .003; overall: p= .054). Comparison of boy and girl controls or cases yielded no recognizable significance (control: p= .044; cases: p=.75).

The VNTR found in the 3'UTR of *SLC6A3* has frequently been associated with ADHD[36-41], but this study found no significant association between ADHD and the VNTR in either total cases or boys alone. When we examined the association using only girls, we found a significant ADHD association with the "not 10" allele, which was also

seen when we restrict our sample to only Caucasians (total: p= .046; boys: p= .82; girls: p= .003). The difference between boy and girl controls or cases did not reach significance at our reduced alpha level (control: p= .039; cases p= .51).

ADRA2A.

Three polymorphisms found in the *ADRA2A* gene were tested (Table 15). For the *Msp*I RFLP, no significant association was found in the total sample or boys alone case-control groups, although the effect was near significance for girls. Restricting our samples to only Caucasians showed no significance (total: p= .83; boys: p= .89; girls: p= .16). The *Hha*I RFLP produced no significant associations in any group. Our previous study found ADHD linked to the DraI polymorphism in the 3'UTR of *ADRA2A* using TDT analysis[143]. In the present study, which contains additional samples, we found similar results (Table 15 footnote), and in addition, we found that the case/control comparison approached significance in the total sample. However, there was a strong association of ADHD with the *Dra*I polymorphism in girls. Similar findings were seen in the Caucasian-only subset (total: p= .25; boys: p= .72; girls: p= .002). The rarer T allele of *Dra*I polymorphism appears to confer risk to develop ADHD primarily in girls. Examining girls versus boys with ADHD yielded significance (p= .009), while boy versus girl controls did not (p=.027) considering the significance level as p-value of .01.

Table 13. Case-control association of *DRD4* (controls vs ADHD all types).

Polymorphism	Sample		No of individuals with genotypes (percentages of genotype)			p-value
Insertion/			I/I	I/D	D/D	
Deletion	Total	Controls	33(48.5)	30(44.1)	5(7.4)	.003*
		Cases	89(73.0)	26(21.3)	7(5.7)	
	Boys	Controls	22(50.0)	20(45.5)	2(4.5)	.002*
		Cases	63(75.9)	14(16.9)	6(7.2)	
	Girls	Controls	11(45.8)	10(41.7)	3(12.5)	.14
		Cases	26(66.7)	12(30.8)	1(2.6)	
VNTR			~7/~7	~7/7	7/7	
	Total	Controls	38(55.1)	28(40.6)	3(4.3)	.76
		Cases	73(60.3)	44(36.4)	4(3.3)	
	Boys	Controls	20(44.4)	23(51.1)	2(4.4)	.16
		Cases	52(61.9)	29(34.5)	3(3.6)	
	Girls	Controls	18(75.0)	5(20.8)	1(4.2)	.28
		Cases	21(56.8)	15(40.5)	1(2.7)	

Allele frequencies for Insertion/deletion polymorphism (I = .71, D= .29), for VNTR (7 repeat = .25, not 7 repeat = .75). No deviation from Hardy Weinberg equilibrium was found in the total controls.

Table 14. Case-control association of *SLC6A3* (controls vs ADHD all types).

Polymorphism	Sample		No of individuals with genotypes (percentages of genotype)				P-value
Exon9 SNP			A/A	A/G	G/G	Sum	
Rs6347	Total	Controls	39(55.7)	27(38.6)	4(5.7)	70	.82
		Cases	66(53.2)	48(38.7)	10(8.1)	124	
	Boys	Controls	22(48.9)	19(42.2)	4(8.9)	45	.66
		Cases	48(56.5)	29(34.1)	8(9.4)	85	
	Girls	Controls	17(68.0)	8(32.0)	0(0.0)	25	.16
		Cases	18(46.2)	19(48.7)	2(5.1)	39	
Intron9 SNP			A/A	A/G	G/G	Sum	
*PflF*I RFLP	Total	Controls	56(80.0)	13(18.6)	1(1.4)	70	.046
*Tth111*I RFLP		Cases	78(62.9)	42(33.9)	4(3.2)	124	
	Boys	Controls	32(71.1)	12(26.7)	1(2.2)	45	.74
		Cases	55(64.7)	27(31.8)	3(3.5)	85	
	Girls	Controls	24(96.0)	1(4.0)	0(0.0)	25	.005*
		Cases	23(59.0)	15(38.5)	1(2.6)	39	
VNTR			10/10	10/~10	~10/~10	Sum	
	Total	Controls	42(60.0)	23(32.9)	5(7.1)	70	.25
		Cases	58(48.3)	54(45.0)	8(6.7)	120	
	Boys	Controls	22(48.9)	19(42.2)	4(8.9)	45	.94
		Cases	42(51.2)	34(41.5)	6(7.3)	82	
	Girls	Controls	20(80.0)	4(16.0)	1(4.0)	25	.010*
		Cases	16(42.1)	20(52.6)	2(5.3)	38	

Allele frequencies: Exon9 SNP (A=0.75, G=0.25), Intron9 SNP (A=0.90, G=0.10), VNTR (10 repeat = 0.76, not 10 repeat (~10) = 0.24). No deviation from Hardy Weinberg equilibrium was found in the controls.

Table 15. Case-control association of *ADRA2A* (controls vs ADHD all types).

Polymorphism	Sample		No of individuals with genotypes (percentages of genotype)				P-value
MspI RFLP			C/C	C/G	G/G	Sum	
Rs1800544	Total	Controls	33(48.5)	26(38.2)	9(13.2)	68	.52
		Cases	50(41.0)	57(46.7)	15(12.3)	122	
	Boys	Controls	20(45.5)	18(40.9)	6(13.6)	44	.95
		Cases	40(47.6)	34(40.5)	10(11.9)	84	
	Girls	Controls	13(54.2)	8(33.3)	3(12.5)	24	.072
		Cases	10(26.3)	23(60.5)	5(13.2)	38	
HhaI RFLP			G/G	G/A	A/A	Sum	
Rs1800545	Total	Controls	50(71.4)	17(24.3)	3(4.3)	70	.43
		Cases	96(77.4)	26(21.0)	2(1.6)	124	
	Boys	Controls	33(73.3)	9(20.0)	3(6.7)	45	.46
		Cases	67(78.8)	16(18.8)	2(2.4)	85	
	Girls	Controls	17(68.0)	8(32.0)	0(0.0)	25	.58
		Cases	29(74.4)	10(25.6)	0(0.0)	39	
DraI RFLP			C/C	C/T	T/T	Sum	
Rs553668	Total	Controls	53(75.7)	15(21.4)	2(2.9)	70	.099[a]
		Cases	75(60.5)	43(34.7)	6(4.8)	124	
	Boys	Controls	32(71.1)	13(28.9)	0(0.0)	45	.25
		Cases	58(68.2)	22(25.9)	5(5.9)	85	
	Girls	Controls	21(84.0)	2(8.0)	2(8.0)	25	.001*[b]
		Cases	17(43.6)	21(53.8)	1(2.6)	39	

a: TDT for the allele T of this SNP is significant as p-values of .039 for control vs ADHD-C (Transmitted: 14, Nontransmitted 5) and .056 for control vs ADHD (C and PI types together) in total (Transmitted: 22, Nontransmitted 11). In boys, the result is not significant, but, in girls, the result is significant, with p-value =.014 for control versus ADHD-C (Transmitted: 6, Nontransmitted 0) & .083 for control vs ADHD-C+PI in total (Transmitted: 9, Nontransmitted 3). Allele frequency in controls: *Msp*I (A=.68, G=.33), *Hha*I (G=.84, A=.16), *Dra*I (C=.86, T=.14). Allele distribution in controls did not differ from Hardy-Weinberg Equilibrium.

Discussion

The results suggest that further scrutiny of potential gender specific genetic correlates of ADHD is necessary. It is unlikely that the associations found in this study were due to population stratification because ethnicity was relatively well-matched between cases and controls, and results generally held when analyses were restricted to Caucasians. However, the small values in each cell lead to a caution that this result needs to be replicated with larger samples. Each of the three studied genes showed different patterns of association with ADHD for boys and girls. The insertion polymorphism in *DRD4* was associated with ADHD in boys, while the G allele of the intron 9 SNP in *SLC6A3* and the T allele of the *Dra*I RFLP of the *ADRA2A* gene were risk alleles in girls. For *SLC6A3* and *DRD4*, there were no significant differences between boy versus girl cases or controls, although for *SLC6A3* there was a trend toward significance in control boys versus girls. Especially for the *SLC6A3* intron 9 SNP and VNTR, the genotypes of control girls are quite different from total controls. This might be due to a protective effect of common homozygotes rather than a causative effect of rare homozygotes. In other words, if rare homozygotes are causative and tightly linked to ADHD, then the most affected children will carry the rare causative allele and most cases would be rare homozygotes of the rare allele. However, if common homozygotes are highly protective against ADHD, most controls would be homozygotes for the common allele. The similar phenomenon is shown in *ADRA2A Dra*I RFLP. Although this effect could depend on the distribution of population with ADHD symptoms, this is one probable explanation for these phenomena. For the *ADRA2A* gene there was a significant difference in allele frequency when comparing girls versus boys with ADHD.

For *DRD4*, we found that the main association to ADHD was with the insertion

polymorphism, which was also reported by McCracken, et al.[131]. For *ADRA2A*, the T allele of the *DraI* polymorphism was associated with ADHD as we reported previously[143]. However, most previous studies of the dopamine transporter, *SLC6A3*, found that ADHD was associated with the 10 repeat allele of the 3' VNTR[35,36]. In contrast, our sample population showed that the 9 repeat version of the VNTR on *SLC6A3* was associated with ADHD. Swanson, et al. also found the 9 repeat allele was transmitted more frequently to ADHD children in a sample of methylphenidate responders[45]. There may be different subtypes for the 9 or 10 repeat alleles[178-180]. For example, differential expression of two subtypes of the 10 repeat allele has been demonstrated[178-180]. As discussed further in the last chapter, different subtypes of the allele may generate different effects. Other possibilities include different linkage disequilibrium between a causative allele and the 9 repeat allele in our sample population, or a gender specific effect of the 9 repeat allele considering the relatively high proportion of girls in our study, or both.

Usually, Bonferroni correction for the multiple testing is too conservative to apply[181]. In this case, the strict Bonferroni p-value which reaches the .05 significant level is .002084. Even considering this p-value, the I/D polymorphism of *DRD4* in boys and the *DraI* RFLP of *ADRA2A* in girls still remains significant. Regarding this correction, the significance of *SLC6A3* might come out during a multiple testing. There should be an alpha-inflation correction procedure, but it is still questionable that the strict Bonferroni correction is appropriate here.

With the present findings that the genetic etiology of ADHD may be different in girls and boys, there are clear gender differences with respect to the catecholmine system. In a rat study, the mRNA level and density of the dopamine transporter was significantly higher in females than in males[78]. Also, in a human study, SPECT results show the

significantly higher density of the dopamine transporter in the striatal region of females[83]. For DA management, females have greater striatal DA release and re-uptake than males in a rat study[182]. A more recognizable result is significant gender and hemisphere differences in mouse development with overall higher DA level in females[183]. Interestingly, when compared to appropriate controls, ADHD children have higher dopamine accumulation in the right midbrain[184], while ADHD adults have lower dopamine decarboxylase activity in the medial and left prefrontal areas[185]. Moreover, several studies have shown that ADHD adults have higher dopamine transporter levels[110,112]. In ADHD adults methylphenidate treatment initially increases striatal dopamine transporter activity followed by a reduction in activity after 4 weeks of treatment[113]. Similar down regulation of the dopamine transporter and the post-synaptic dopamine receptor in striatum was seen in ADHD boys after three months of treatment with methylphenidate[186]. It is not known how this down regulation occurs, but it is possible that the dopamine system automatically adjusts to the consistent higher level of synaptic dopamine caused by methylphenidate treatment. Regulatory control of these components of the catecholamine system may operate differently in males and females.

ADHD has been described as an inhibition dysfunction. The normal inhibition process involves prefrontal lobe activation, catecholaminergic transmission from frontal lobe to striatum, information processing in the striatum, and retransmission from the striatum. Dysfunction of this prefrontal-striatal network has been implicated in the etiology of ADHD. Taken together with neuroimaging studies, the suspected etiology of ADHD is low dopaminergic transmission from the prefrontal lobe to the striatum along with low striatal activity through high accumulation of dopamine in the striatum, creating inadequate inhibition through the adrenergic and GABA systems. *ADRA2A* is closely

related to the dopamine system. Clonidine, the *ADRA2A* agonist sometimes used in ADHD treatment, is known to reduce dopamine and increase GABA in the nucleus accumbens[187].

Previous studies suggest that dopamine level is important for proper prefrontal-striatal function in both girls and boys, but that females may have better systematic management of high levels of dopamine as shown in animal studies[173,182,183]. In managing larger levels of dopamine in girls, a speculation is that with more active dopamine transporters, higher synaptic release of dopamine, and probably lower dopamine receptors in response to released dopamine, a polymorphism that regulates expression of the dopamine transporter may be more effective in deepening ADHD symptoms than a polymorphism that regulates expression of the dopamine receptor (Figure 4). Boys may need more dopamine receptors due to reduced release ability of DA in the synapses, and a polymorphism that regulates expression of the dopamine receptor would cause more differences in the receptor level between the boys who have the polymorphism and the boys who do not have the polymorphism.

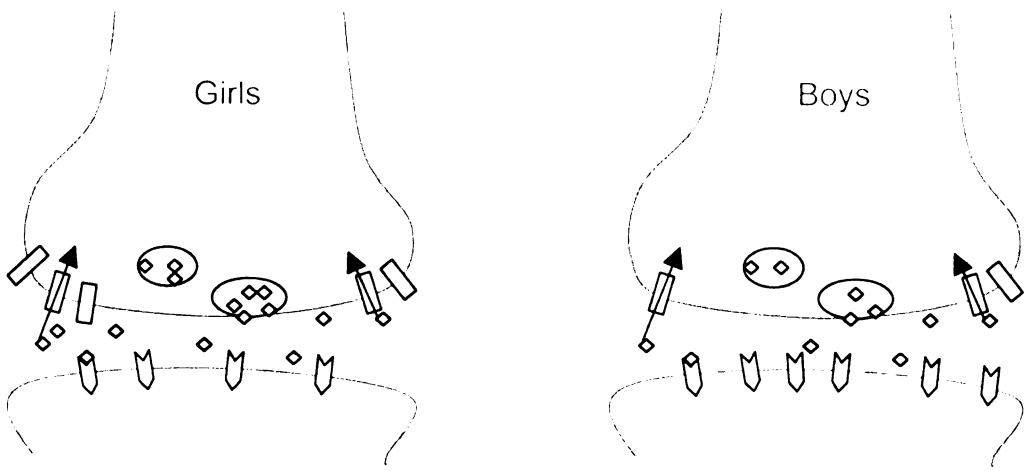


Figure 4. Possible schematic diagram of synapses of girls and boys.

(rectangles: dopamine transporter, small squares: dopamines, and arrows: dopamine receptors)

This speculation is consistent with our finding that the dopamine transporter polymorphism is significantly associated with ADHD in girls rather than boys, whereas the dopamine receptor polymorphism is more significantly associated with ADHD in boys than in girls. Also, higher dopamine levels in women may require more α-2A-adrenergic receptors for adequate regulation of dopamine levels, particularly in the nucleus accumbens. This agrees with our result that the polymorphism responsible for the expression of those receptors was more significantly associated with ADHD in girls than in boys.

Conclusion

Overall, quantitative measurements of inattentiveness and hyperactivity based on DSM-IV are recommended and gender difference needs to be considered in follow-up studies. One major concern of the present study is the relatively small sample sizes that result from splitting the genders or DSM-IV subtypes. Further studies need larger sample populations to confirm the findings in this chapter.

Chapter 4

Haplotype Analysis

Introduction

Based on the initial haplotype analysis of *ADRA2A*, it is clear that LD between markers is a very important criterion for marker selection in the candidate gene if the gene region is in high linkage disequilibrium between polymorphisms. For an example of two tagging SNPs with allele A (frequency .7) and allele B (frequency .8), if D' and r^2 are both high, haplotype frequencies would be .7 for AB, .2 for ab, and .1 for aB. If D' is high and r^2 is low, haplotype frequencies could be .5 for AB, .3 for aB, and .2 for Ab. Therefore, SNPs with a high value of D' and low value of r^2 are better for capturing the possible haplotypes in the gene region. In this chapter, three more SNPs farther along the gene region are selected to find such polymorphisms which may cover additional possible haplotypes.

The two other candidate genes, *SLC6A3* and *DRD4* show moderate and low linkage disequilibrium, respectively, as shown in the literature review in chapter 2. In examining associations between genes and the disorder, high linkage disequilibrium between markers may not be necessary[188]. Bearing in mind the purpose is to find associations between genes and the disorder, screening all the polymorphisms in candidate genes would be unnecessarily costly in time and money, therefore the SNPs are selected based on the spacing and polymorphic status.

In dealing with unambiguous phases in haplotype construction, the program, PHASE, is used. PHASE implements methods for estimating haplotypes from population genotype data using a Bayesian statistical method.[189,190] Using PHASE, case-control

association can be measured in terms of expected frequencies of haplotypes. On the other hand, the program UNPHASED tests the haplotype-based TDT considering transmission as a case-control situation[191]. Reconstructing haplotypes from PHASE may not be highly reliable in further applications due to the magnification of statistical error; therefore, case-control tests and QTDTs of individual polymorphisms are conducted for comparison. Recognizing the better detection through QTDT than TDT, QTDTs are primarily considered in this chapter.

Updated haplotype analysis for *ADRA2A*

ADRA2A is located in 10q25.2, near the middle of chromosome 10 and the gene size is 3,649 bp with one exon. The gene region from 10,000 bp upstream to 2,000 bp downstream contains a total of 19 SNPs. After screening the gene region for polymorphic status, three SNPs were typed. Because the previous LD study showed significant D' within all three typed SNPs, it is not known how far the linkage disequilibrium extends beyond the gene region. Three more SNPs were selected for the haplotype analysis of *ADRA2A* based on the spacing and availability for the assays. The brief summary of selected SNPs is in Table 16. The first two SNPs, rs638019 and rs491589, are validated and inventoried assays by Appliedbiosystems (ABI), and the last SNP is non-inventoried yet functionally tested assay. All markers are in Hardy-Weinberg equilibrium among founders.

Table 16. SNP summary in *ADRA2A*.

Assay ID	c996421	c996423	*Msp*I	*Hha*I	*Dra*I	c3181571
rs #	rs638019	rs491589	rs1800544	rs1800545	rs553668	rs602618
Public position	112821869	112824612	112826493	112827528	112829569	112833075
distance b/w	2743	1881	1035	2041	3506	
Relative position	1	2744	4625	5660	7701	11207
allele frequency[a]	.30	.20[c]	.26	.11	.19	.42[c]
allele frequency[b]	.31	.15	.30	.11	.18	.31

a: minor allele frequency available from ABI; b: minor allele frequency of all parents; c: minor allele frequency from NCBI.

As shown in Table 17, the linkage disequilibrium is very high near the *ADRA2A* gene region. There is one SNP, *Hha*I, that shows low r^2 with other SNPs. The graphical summary using GOLD shows this more clearly (Fig 5). Red color represents high LD, while blue represents low LD. The blue region near *Hha*I of r^2 plot as well as the LD table indicates that the minor allele of *Hha*I is usually linked to the minor allele of c996421, *Msp*I, and c3181571, but to the major allele of c996423 and *Dra*I. Except for *Hha*I, most SNPs are linked to each other through their minor alleles. One interesting feature of this LD map is that minor alleles of SNPs are sorted with the minor alleles of either HhaI *or* c996423 and DraI, which is unexpected if the frequencies of those SNPs are considered with the allele age.

Table 17. Linkage disequilibrium in *ADRA2A*.

D' (r^2)	c996421	c996423	*Msp*I	*Hha*I	*Dra*I	c3181571
c996421	-	.92 (.35)	.93 (.73)	.91 (.19)	.95 (.45)	.90 (.77)
c996423		-	.79 (.30)	.82 (.01)	.97 (.77)	.89 (.34)
*Msp*I			-	.89 (.22)	.83 (.39)	.91 (.75)
*Hha*I				-	.92 (.02)	.82 (.16)
*Dra*I					-	.91 (.43)
c3181571						-

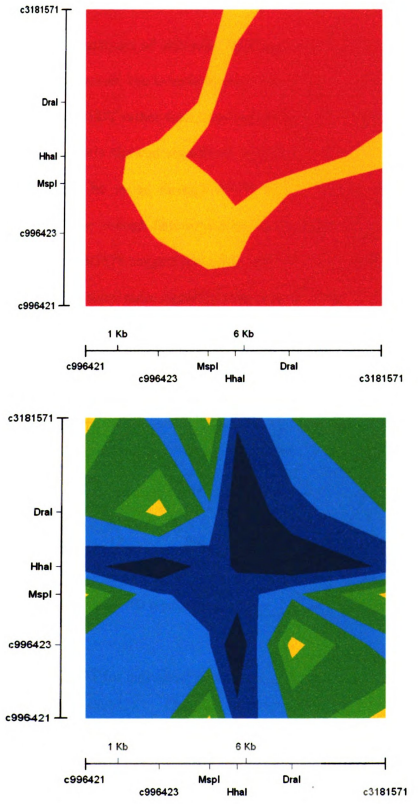


Figure 5. Graphical summary of LD in *ADRA2A*.

The analyses of individual SNPs are listed below in Table 18 and 19. Similar to the previous result, the association between ADRA2A and ADHD was more sensitively detected in QTDT rather than case-control association tests. As expected from previous results, the girls showed significant associations with ADHD compared to boys. Most SNPs cannot be tested through QTDT due to smaller samples, but MspI RFLP and rs602628 have enough heterozygosity so that some QTDTs are tested. The significant results for rs602628 suggest that other SNPs, such as rs638019, rs491589, and DraI RFLP, will probably show more significant results with more girl samples.

Table 18. Case-control association tests for individual SNPs on *ADRA2A*.

Assay ID rs #	C996421 rs638019	C996423 rs491589	*Msp*I rs1800544	*Hha*I rs1800545	*Dra*I rs553668	c3181571 rs602628
Total dx0	.91	.057	.52	.43	.099	.85
Total dx1	.95	.068	.40	.55	.079	.95
Total dx2	.73	.14	.98	.52	.58	.61
Boys dx0	.35	.39	.95	.46	.25	.16
Boys dx1	.3	.48	.98	.60	.27	.15
Boys dx2	.91	.17	.81	.51	.18	.70
Girls dx0	.12	.008	.072	.58	.001	.080
Girls dx1	.011	.002	.012	.38	.0003	.007
Girls dx2	.88	.10	.71	.93	.035	.81

dx0: control vs all ADHD types; dx1: control vs ADHD-C; dx2: control vs ADHD-PI.

Table 19. QTDT for individual SNPs on *ADRA2A*.

Assay ID rs #	c996421 rs638019	C996423 Rs491589	*Msp*I rs1800544	*Hha*I rs1800545	*Dra*I rs553668	C3181571 rs602628
Total ATTN	.0039	.0043	.0991	NS	.0282	.0792
Total HYP	.0065	.0263	NS	NS	.0382	NS
Boys ATTN	NS	.0849	NS	NS	NS	NS
Boys HYP	NS	NS	NS	NS	NS	NS
Girls ATTN	NT	NT	.0599	NT	NT	.0050
Girls HYP	NT	NT	NT	NT	NT	.0013

Table 20. Haplotype association tests through PHASE.

Samples	Index[a]	haplotype	E(freq)	E[Freq(controls)]	E[Freq(cases)]
Total .02	1	211111	.619	.636	.610
	2	122122*	.146	.069	.190
	3	112212	.130	.151	.118
	4	112122	.037	.065	.021
	5	111111	.019	.017	.020
	6	112112	.014	.025	.008
	7	211112	.013	.011	.014
Boys .17	1	211111	.636	.606	.651
	2	122122*	.136	.078	.166
	3	112212	.121	.141	.110
	4	112122	.036	.067	.019
	5	111111	.027	.036	.022
	7	211112	.014	.023	.009
	6	112112	.012	.013	.012
Girls .03	1	211111	.578	.670	.519
	2	122122*	.167	.055	.240
	3	112212	.137	.157	.125
	4	112122	.038	.063	.023
	7	211112	.020	.006	.029
	8	112121	.019	.002	.030
	6	112112	.015	.035	.002

Estimated haplotype frequencies are listed (E(freq)) and p-values are from 100 permutations. a: Numbers were marked in the order of the haplotype frequencies from total samples.

Haplotype analysis was done using all the SNPs typed because all SNPs are linked to each other showing high LD in terms of D'. PHASE gives a p-value of .02 for testing the significant differences in haplotype frequencies of total samples through default 100 permutations. The ADHD phenotypes used in this analysis contain both ADHD-C and ADHD-PI. As expected for the significant association of ADHD girls and *ADRA2A*, the p-value was .17 for boys and .03 for girls. The haplotype frequencies which account for more than 98% of the total possible haplotypes are summarized in Table 20. As indicated (*), the significance comes mostly from the frequency difference of haplotype 122122 between controls and cases in all tests: total, boys, and girls. Compared

to the individual test result of each SNP, all the alleles in the haplotype 122122 are associated causatively with ADHD. Although the constitution of SNPs is different, the frequencies of haplotypes are not much different from the reported haplotype frequencies also using PHASE.[192]

UNPHASED gives less significant associations than the association test using individual SNPs similar to the haplotype analyses using PHASE (Table 21). The results of individual SNPs using UNPHASED are analogous to the results of QTDT with the same pattern but different p-values. The haplotype analysis confirms the causative association between the haplotype 122122 and ADHD. Interestingly, the significant association with ADHD girls is due to not only the causative effect of the haplotype 122122, but also the protective effect of another haplotype, 211111. The protective haplotype consists of opposite alleles of the causative haplotype, 122122, except the HhaI RFLP.

Table 21. Haplotyope association tests through UNPHASED.

Samples	Tests	Global p-value[a]	Associated haplotype[b]	Direction	p-value of the haplotype
Total	Inattentiveness	.024	1-2-2-1-2-2	Causative	.009
	Hyperactivity	.13	1-2-2-1-2-2	Causative	.026
Boys	Inattentiveness	.62	1-1-2-1-2-2	-	.095
	Hyperactivity	.64	1-1-2-1-2-2	-	.10
Girls	Inattentiveness	.004	2-1-1-1-1-1	Protective	.004
			1-2-2-1-2-2	Causative	.016
	Hyperactivity	.012	2-1-1-1-1-1	Protective	.005
			1-2-2-1-2-2	Causative	.007

a: permutated 1000 times. b: p-values less than .05 or most associated haplotype.

The individual and haplotype association test verified again the association between the ADRA2A gene and ADHD. Through the individual and haplotype association, the haplotype 122122 is associated with ADHD in a causative way possibly due to the contribution of each polymorphism. The protective effect of the haplotype 211111 in the UNPHASED result is possibly due to the protective effect of the haplotype in girls considering lower *p*-values than the causative haplotype. Yet, it should be noted that this most common haplotype, 211111, could be paired often with the causative haplotype so to make higher significant association between the haplotype 211111 and ADHD in girls.

Haplotype analysis of *SLC6A3*

SLC6A3 is located in the telomeric region of chromosome 5 and is much larger in size than *ADRA2A* or *DRD4*. As mentioned in chapter 2, this gene has been previously examined in association studies and a quite dense LD map is already available. A previous study on linkage disequilibrium showed two haplotype blocks in the gene region, one from the promoter to intron 6, the other from exon 9 to the 3'UTR[58]. In our initial study, 4 SNPs and a 3'UTR VNTR were typed by PCR, RFLP, or sequencing. The four SNPs are the exon 9 non-synonymous amino acid change SNP, the intron 9 SNP, and two exon 15 3'UTR SNPs (Figure 6; indicated with solid arrows). All those polymorphisms showed relatively high linkage disequilibrium, as expected from the previous study. With the VNTR as a center, two SNPs of exon 9 and intron 9 *and* two 3'UTR SNPs showed higher linkage disequilibrium with respect to their pairwise close locations.

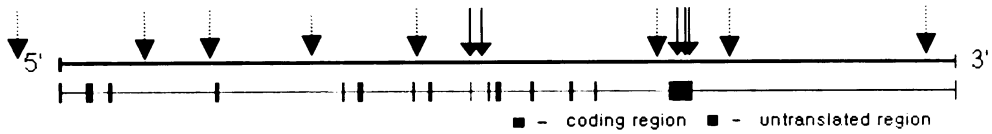


Figure 6. Gene structure and genotyped polymorphisms of *SLC6A3*.

Arrows from left to right indicate polymorphisms depending on marker numbers shown in table 22.

Table 22. Polymorphism summary in *SLC6A3*.

Marker #	Assay ID	rs #	Public position	Distance b/w	Relative position	Allele freq[a]	Allele freq[b]
1	C27477615	rs3756450	1501148	9794	1	.16	.15
2	C2960958	rs403636	1491354	5478	9795	.22[c]	.17
3	C3284838	rs465130	1485876	8971	15273	.19	.23
4	C3284822	rs464049	1476905	7763	24244	.49	.44
5	C2396880	rs40358	1469142	4730	32007	.12	.15
6	Dat1E9	rs6347	1464412	1427	36737	.19	.26
7	Dat1I9	rs8179029	1462985	14908	38164	.09[c, e]	.16
8	C2960969	rs40184	1448077	958	53072	.39	.44
9	VNTR	-	1447119	775	54030	.68-83[d]	.76
10	3'UTR SNP1	rs3797200	1446344	383	54805	-	.22
11	3'UTR SNP2	rs1809939	1445961	1592	55188	.21[c]	.22
12	C2854709	-	1444369	6371	56780	.33	.36
13	C2854700	-	1437998	-	63151	.17	.13

a: minor allele frequency available from ABI; b: minor allele frequency of parents; c: minor allele frequency from NCBI; d: various source from publications; e: published reports of the frequency is similar to the frequency of this sample population[37].

Additional SNPs were typed by Taqman genotyping assays. The validated assays were selected primarily, and further SNPs were selected based on their spacing. The locations are indicated in Figure 6 with dotted arrows. The information of all the

polymorphisms is summarized in Table 22. All markers were checked for Hardy-Weinberg equilibrium among founders, and markers show no deviation.

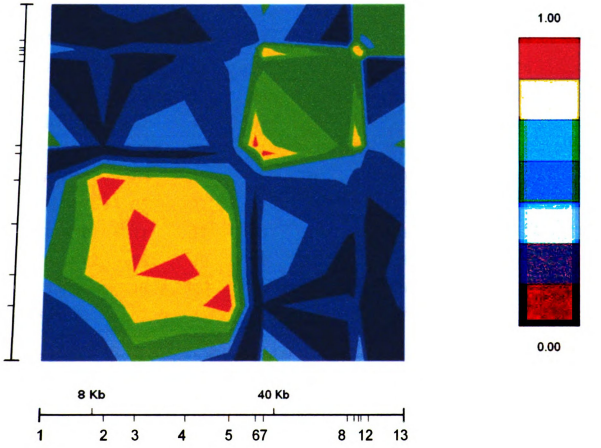
Table 23. Linkage disequilibrium in *SLC6A3*.

D'	2	3	4	5	6	7	8	9	10	11	12	13
1	.15	.46	.36	.17	.04	.52	.02	.27	.41	.43	.13	.02
2	-	.84	.79	.95	.11	.13	.20	.17	.15	.09	.07	.16
3		-	.88	.82	.03	.37	.06	.06	.27	.27	.10	.19
4			-	.79	.001	.43	.17	.16	.28	.25	.02	.19
5				-	.18	.18	.30	.19	.18	.16	.22	.21
6					-	.93	.44	.45	.41	.44	.22	.38
7						-	.55	.80	.76	.74	.37	.45
8							-	.50	.42	.43	.03	.13
9								-	.82	.80	.50	.51
10									-	.88	.47	.48
11										-	.41	.52
12											-	.45
13												-

As indicated in Table 23 and Figure 7, there are two LD blocks in this gene region. This LD structure is similar to the LD studies from others which are summarized in Chapter 2. A further SNP (marker 1) beyond 3'UTR region was not included in the first block indicating the sizes of the block. The last SNP (marker 13) shows some LD with the second blocks, but the LD is decayed due to the SNP, rs40484 (marker 8, ID c2960969). Considering the high minor allele frequency of the SNP (.44), it is possible that this SNP is a neutral and old polymorphism showing relatively low LD with nearby SNPs. Unlike *ADRA2A*, in which the HapMap project has only one typed SNP, the

SNPs. Unlike *ADRA2A*, in which the HapMap project has only one typed SNP, the available LD results on the *SLC6A3* region were similar for the five SNPs that are typed in this study and the HapMap. Based on this LD structure, the haplotypes using all polymorphisms as well as two blocks (markers 2-5 and markers 6-13) were selected for haplotype analyses.

Figure 7. Graphical summary of LD in *SLC6A3* (D').



Case-control associations for individual polymorphisms are summarized in Table 24. Most SNPs located in the second LD block are associated with ADHD in the case-control association tests. Similar to the pattern in chapter 3, girls show more significant

with both ADHD-C and ADHD-PI, but c2960969 is associated mostly with ADHD-PI and the polymorphisms, VNTR, 3'UTR SNP1 and 2, c2854709, *and* c2854700, are associated mostly with ADHD-C.

Table 24. Case-control association tests for individual polymorphisms on *SLC6A3*.

Assay ID	Total dx0	Total dx1	Total dx2	Boys dx0	Boys dx1	Boys dx2	Girls dx0	Girls dx1	Girls dx2
C27477615	.51	.49	.63	.59	.68	.65	.58	.39	.76
C2960958	.65	.47	.86	.31	.087	.20	.88	.67	.63
C3284838	.59	.67	.22	.61	.72	.50	.87	.69	.43
C3284822	.67	.63	.76	.50	.65	.39	.96	.76	.49
C2396880	.70	.70	.83	.42	.17	.16	.93	.61	.66
Dat1E9	.82	.67	.88	.66	.66	.86	.16	.10	.35
Dat1I9	.046	.060	.11	.74	.72	.71	.005	.007	.004
C2960969	.019	.11	.009	.46	.49	.11	.007	.058	.019
VNTR	.25	.32	.32	.94	.83	.25	.01	.002	.39
3'UTR SNP1	.11	.17	.25	.78	.96	.17	.028	.011	.23
3'UTR SNP2	.06	.10	.17	.58	.83	.11	.028	.011	.23
C2854709	.10	.033	.53	.33	.28	.91	.049	.019	.083
C2854700	.57	.45	.40	.53	.44	.85	.43	.86	.23

dx0: control vs all ADHD types; dx1: control vs ADHD-C; dx2: control vs ADHD-PI.

With similar patterns to the results of previous chapters, the QTDT results for individual SNPs in *SLC6A3* show no association between any SNP and ADHD except rs40184 (ID c2960969). C2960969 showed borderline significance in inattentiveness with p-value of .035 in total samples. ADHD girls are more significantly associated with c2960969 (p-values: .0034 for inattentiveness and .030 for hyperactivity).

Haplotype analyses using PHASE reaffirm the association between *SLC6A3* and ADHD girls and total samples in the second LD block. Similar to the results in *ADRA2A*, the haplotype analysis is not as sensitive as the association tests using individual polymorphisms (Table 25). The haplotype frequencies consisting of all polymorphisms are summarized in Table 26 for total samples and girls. The "2" in the haplotypes represents 10 repeats of VNTR, and "3" represents 9 repeats of VNTR. The haplotypes from total samples in Table 26 cover only 50 % of all haplotype frequencies with various haplotypes with the frequency of around .02. Unlike *ADRA2A*, there is no haplotype which shows obvious differences in frequencies among listed haplotypes.

Table 25. p-values of the case-control haplotype analyses using PHASE.

PHASE results	All polymorphisms	LD block 1	LD block 2
Total	.06	.92	.05
Boys	.34	.42	.38
Girls	.04	.87	.003*

Permutated 100 times. *: permutated 1000 times.

The results from the second LD block show more obvious differences between controls and cases (Table 27). The listed haplotypes of total samples cover 85% of all possible haplotypes. The haplotype 221 3 2212 is associated with ADHD in both total samples and girls. Minor differences are; a) the most frequent haplotype, 112 2 1122, is more frequent in controls suggesting possible protective effect of the haplotype; b) the haplotype 222 3 2212 is associated with ADHD in total samples, whereas the haplotype 221 3 2222 is associated with ADHD in girls suggesting that the marker *Dat1*9 does not have much effect in total samples, whereas C2854709 may not be effective in girls.

Table 26. Summary of haplotype frequencies with all polymorphisms.

Samples and p-values	Index*	haplotype	E(freq)	E[Freq(controls)]	E[Freq(cases)]
Total .06	1	12121112 2 1122	.131	.149	.121
	2	12121112 2 1112	.063	.051	.070
	3	12121112 2 1121	.063	.044	.073
	4	12211112 2 1122	.035	.024	.042
	5	12111111 2 1122	.035	.026	.040
	6	12121221 3 2212	.034	.027	.038
	7	12111211 2 1122	.031	.039	.026
	8	11122221 3 2222	.030	.018	.036
	9	12111111 3 2212	.029	.025	.031
	10	11122112 2 1122	.026	.031	.024
	11	12111112 2 1122	.025	.036	.018
Girls .04	1	12121112 2 1122	.184	.247	.143
	2	12121112 2 1112	.059	.049	.066
	3	12121112 2 1121	.049	.029	.062
	10	11122112 2 1122	.039	.050	.031
	11	12111112 2 1122	.036	.055	.023
	12	22211112 2 1122	.034	.040	.030
	16	12121111 3 2212	.025	.033	.020
	4	12211112 2 1122	.025	.039	.016
	15	12211112 2 1112	.023	.028	.019
	6	12121221 3 2212	.021	.005	.032
	5	12111111 2 1122	.017	.006	.025

Permutated 100 times. *: Numbers were marked in the order of the haplotype frequencies from total samples.

Table 27. Summary of haplotype frequencies with polymorphisms in the second LD block.

Samples	Index[a]	haplotype	E(freq)	E[Freq(controls)]	E[Freq(cases)]
	1	112 2 1122	.266	.301	.246
	2	112 2 1112	.113	.113	.113
	3	111 2 1122	.093	.082	.098
	4	112 2 1121	.088	.067	.099
Total	5	221 3 2212*	.072	.040	.090
.05	6	111 3 2212	.064	.073	.059
	7	221 3 2222	.051	.038	.057
	8	211 2 1122	.048	.060	.041
	9	111 2 1121	.038	.030	.043
	10	222 3 2212*	.021	.003	.031
	1	112 2 1122*	.327	.462	.240
	2	112 2 1112	.138	.132	.143
	4	112 2 1121	.077	.064	.085
	6	111 3 2212	.055	.053	.056
Girls	5	221 3 2212*	.053	.008	.082
.003[b]	3	111 2 1122	.047	.041	.051
	8	211 2 1122	.040	.050	.033
	7	221 3 2222*	.039	.007	.059
	11	111 2 1112	.030	.010	.043
	15	112 2 1111	.022	.018	.024

Permutated 100 times. a: Numbers were marked in the order of the haplotype frequencies from total samples; b: permutated 1000 times.

Unexpectedly, the haplotype analyses using UNPHASED reveal an association between ADHD and *SLC6A3*. As shown in Table 29, the associated haplotype is 1-2-1-2-1-1-1-2-2-1-1-2-2, which is the most frequent haplotype in PHASE results. In the second LD block, the associated haplotype, 1-1-2-2-1-1-1-2, is mostly a part of the haplotype 1-2-1-2-1-1-1-2-2-1-1-2-2 except for the SNP C2854709. An interesting feature is that this haplotype is protective and the most common haplotype. Moreover, the global p-values are not significant before permutations possibly suggesting the possible effects of not only the protective haplotype itself, but also many causative rare haplotypes, which are paired with this haplotype in each individual. No results of the first LD block show

association using either PHASE or UNPHASED.

Table 28. p-values of the association tests from UNPHASED.

p-values	polymorphisms	ATTN	HYP
Total	all	.016	.39
	LD1	.46	.76
	LD2	.47	.14
Boys	all	.5	.016
	LD1	1	1
	LD2	.25	.033
Girls	all	.059	.99
	LD1	.48	1
	LD2	.006	.18

Permutated 1000 times.

Table 29. Haplotyope association tests through UNPHASED.

Samples	Tests		Global p-value[a]	Associated haplotype[b]	Direction	p-value of the haplotype
Total	ATTN	All	.016	1-2-1-2-1-1-1-2-2-1-1-2-2	protective	.017
		LD2	.47	-	-	-
	HYP	All	.39	-	-	-
		LD2	.14	-	-	-
Boys	ATTN	All	.50	-	-	-
		LD2	1.00	-	-	-
	HYP	All	.016	1-2-1-2-1-1-1-2-2-1-1-2-2	protective	.020
		LD2	.033	1-1-2-2-1-1-1-2	protective	.013
Girls	ATTN	All	.059	1-2-1-2-1-1-1-2-2-1-1-2-2	protective	.073
		LD2	.006	1-1-2-2-1-1-2-2	protective	.0155
	HYP	All	.99	-	-	-
		LD2	.18	-	-	-

a: same as Table 24; b: only listed in case of a significant global p-value.

The association tests of individual polymorphisms as well as haplotypes reconfirm the association between ADHD and *SLC6A3* mostly through the second LD block. Again, girls show more associations between ADHD and this gene, but the TDT results for haplotypes of boys show the possible association between hyperactivity and *SLC6A3* in a protective way.

Haplotype analysis of *DRD4*

DRD4 is 3398 bp consisting of four exons located in the telomeric region of chromosome 11. As described in chapter 2, the promoter region of this gene represents a possible recombination spot in another study, and several evolutionary investigations on this gene show an LD decay from 7 repeats of VNTR as a center. The SNP selection for *DRD4* is similar to *SLC6A3* and *ADRA2A*. Some of the selected SNPs do not show enough heterozygosity to be analyzed, and several of them did not work well in the case of the not-validated but functionally tested assays. Finally, four validated assays and two functionally tested assays were successfully typed and analyzed. Further typing in the region between the first two assays and the promoter is not considered because the region is too far from the gene region. All markers are in Hardy-Weinberg equilibrium among founders except c7470701, which cannot be tested reliably due to the small values in a cell because of the low frequency.

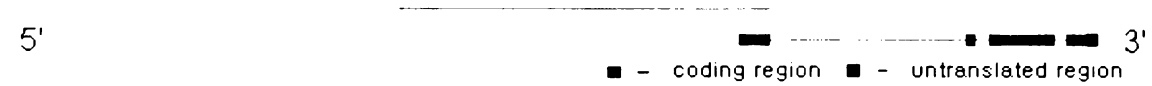


Figure 8. Gene structure and typed polymorphisms.

Table 30. Summary of typed polymorphism in *DRD4*.

Marker #	Assay ID	rs #	Public position	Distance b/w	Relative position	Allele freq[a]	Allele freq[b]
1	C1611535	-	615085	610	1	.37	.24
2	C1611534	-	615695	10504	611	.26	.32
3	C7470692	rs936460	626199	297	11115	.28[c]	.32
4	C7470693	rs936461	626496	433	11412	.47	.41
5	C7470701	rs916455	626929	915	11845	.07[c]	.03
6	In / Del	rs4646983	627844	4143	12760	.06[c, e]	.21
7	VNTR[f]	-	631987	2761	16903	~.19[d]	.20
8	C25652468	-	634748	-	19664	.13	.07

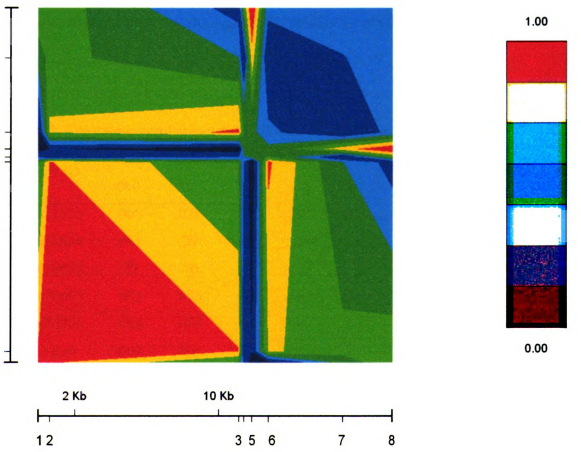
a: minor allele frequency available from ABI; b: minor allele frequency of parents calculated by merlin; c: minor allele frequency from NCBI; d: various source from publications; e: the minor allele frequency is around .19 in published results[131]; f: frequency of 7 repeats.

As summarized in Table 31 and Figure 9, the promoter region of this gene represents relatively low LD between markers which are close. It is suggested that this low LD region is a recombination hot spot and the polymorphisms in this region are associated independently from VNTR[122]. Although markers are not enough to interpret, the LD decays toward the *DRD4* gene region, and, after marker 5 (c7470701), the LD pattern shows inconsistency not depending on the distance. The r^2 values are high only between the first three SNPs, suggesting these SNPs are associated with each other within their minor alleles.

Table 31. Linkage disequilibrium in *DRD4*.

D' (r^2)	2	3	4	5	6	7	8
1	**.88 (.58)**	.57 (.35)	.28 (.06)	.07(.0001)	.09 (.003)	.51	.66 (.02)
2	-	.86 (.46)	.22 (.02)	.15 (.002)	.74 (.05)	.61	.65 (.01)
3		-	.30 (.06)	.05(.0002)	**.88 (.09)**	.43	.23 (.009)
4			-	.49 (.01)	.59 (.13)	.15	.63 (.05)
5				-	.48 (.03)	.81	1.00(.003)
6					-	.15	.33 (.03)
7						-	.40

Figure 9. Graphical summary of linkage disequilibrium in *DRD4* (D').



Individual polymorphisms were tested for case-control association (Table 32). As described in the previous chapter, newly typed SNPs show more significant associations with ADHD boys. Some of the associated polymorphisms show a preference toward a subtype of ADHD like the results in *SLC6A3*. Interestingly, the SNP c1611535 is associated with ADHD especially in ADHD boys, even though this SNP is located far from the gene region. There are two more genes, interferon regulatory factor 7 (*IRF7*) and mucin and cadherin-like (*MUCDHL*) near this SNP. More investigation is necessary for the association of this SNP to figure if other genes or regulatory elements near the SNP are involved in ADHD, or if a functional polymorphism strongly linked to this SNP resides in the *DRD4* region, although this is not likely considering the low LD with other polymorphisms in the gene region.

Table 32. The case-control associations of individual polymorphisms.

Assay ID	Total dx0	Total dx1	Total dx2	Boys dx0	Boys dx1	Boys dx2	Girls dx0	Girls dx1	Girls dx2
C1611535	.005	.029	.038	.003	.009	.088	.59	.76	.45
C1611534	.081	.29	.035	.033	.067	.10	.96	.40	.29
C7470692	.29	.16	.67	.14	.18	.29	.47	.30	.52
C7470693	.082	.075	.39	.047	.067	.063	.37	.40	.63
C7470701	.084	.051	.62	.38	.29	.98	.10	.087	.41
In / Del	.003	.003	.20	.002	.002	.35	.14	.13	.44
VNTR	.76	.99	.18	.16	.29	.11	.28	.14	.66
C25652468	.49	.41	.91	.75	.52	.48	.49	.73	.41

Similar to the results in *SLC6A3*, none of the QTDTs finds associations between ADHD and *DRD4*, except the association between 4 repeats allele of VNTR and

hyperactivity in girls with p-value of .0243. The 4 repeats allele of VNTR is the only one which shows population stratification in the hyperactivity of girls using QTDT program. Considering most polymorphisms are not tested due to the small sample size, it may be possible to find associations through QTDT with an increased sample size.

Haplotype association studies using PHASE are summarized in Table 33. The haplotypes listed in total samples cover 70% of all haplotypes. Although the p-value is .008 in total samples, the haplotype frequencies do not differ between cases and controls suggesting the significance may come from the differences in many haplotypes. The haplotype that shows most obviously a difference between cases and controls is 11221 1 4 2. Depending on the individual association of polymorphisms, boys are expected to show more significant results, but both boys and girls do not reveal associations suggesting the association with total samples may come from many different haplotypes which are effective only in one gender. The haplotype studies consisting of polymorphisms only near DRD4 (markers 3-8) show similar patterns but less significant associations suggesting the significant result of haplotype association comes from the entire region including first two SNPs (p-values: .07 for total samples; .10 for boys; .44 for girls). Differing from the results of *SLC6A3*, none of haplotype results from UNPHASED shows an association between *DRD4* and ADHD as expected from QTDTs of individual polymorphisms.

The gene *DRD4* is associated with ADHD through the case-control associations in both individual polymorphisms and haplotypes. As described in Chapter 3, the results here reinforce the possibility that boys are more inclined toward ADHD because of this gene. Considering the result of total samples in both individual polymorphisms and haplotype associations, girls may be affected by *DRD4*, although the effect may be much

less than in boys.

Table 33. Haplotype association studies using PHASE.

Samples & p-values	Index	haplotype	E(freq)	E[Freq(controls)]	E[Freq(cases)]
Total .008*	1	11111 1 4 2	.326	.253	.367
	2	22221 1 7 2	.091	.107	.082
	3	22211 1 7 2	.070	.062	.075
	4	11121 2 4 2	.050	.064	.043
	5	11121 1 4 2	.045	.039	.048
	6	22221 1 4 2	.036	.033	.037
	7	11111 1 7 2	.027	.025	.028
	8	11221 1 4 2*	.024	.006	.034
	9	11111 2 4 2	.024	.037	.016
	10	21121 2 4 2	.022	.033	.017
Boys .06	1	11111 1 4 2	.344	.260	.387
	2	22221 1 7 2	.106	.148	.084
	3	22211 1 7 2	.065	.058	.069
	4	11121 2 4 2	.042	.046	.039
	6	22221 1 4 2	.031	.029	.032
	9	11111 2 4 2	.027	.041	.020
	10	21121 2 4 2	.027	.047	.017
	7	11111 1 7 2	.025	.022	.027
	11	11111 1 3 2	.025	.000	.038
	5	11121 1 4 2	.024	.023	.024
Girls .48	1	11111 1 4 2	.296	.260	.318
	4	11121 2 4 2	.086	.113	.069
	5	11121 1 4 2	.078	.048	.097
	3	22211 1 7 2	.068	.056	.076
	7	11111 1 7 2	.054	.044	.060
	6	22221 1 4 2	.039	.034	.042
	15	21121 2 8 2	.035	.060	.018
	2	22221 1 7 2	.030	.015	.040
	8	11221 1 4 2	.025	.003	.040
	9	11111 2 4 2	.023	.033	.017

Permutated 100 times. *: permutated 1000 times.

Conclusion

The association studies using both individual polymorphisms and haplotypes reveal these candidate genes are associated with ADHD. Although there are some minor variations depending on the association tests, as shown in chapter 3, *ADRA2A* and *SLC6A3* are associated in ADHD girls, whereas *DRD4* is associated mostly in boys. As indicated in chapter 2, the TDT and case-control association tests show different results.

Haplotype association tests follow in the same manner to the associations of individual polymorphisms in terms of both gender differences and differences in the TDT and case-control associations. However, unlike the expectation in Chapter 1, tests using haplotypes do not consistently show higher detection of the associations than tests using individual polymorphisms. In summary, a set of polymorphisms in an LD block shows better associations and easier interpretation compared to a set of polymorphisms in a low LD region.

Similar to the results in chapter 2, most of endophenotypes show no significant associations with p-values less than .05. The associations with individual polymorphisms are 1) between c2854709 of *SLC6A3* and response variability in girls with p-value of .014; 2) c1611534 of *DRD4* and the stop signal reaction time test in boys with p-value of .038 through the total evidence of association in the QTDT program. Both SNPs are associated with ADHD, but there are several other polymorphisms more strongly associated with ADHD in these gene regions leading to cautions in the endophenotype studies.

Materials and methods

Setting up 384 well plates and quantification

For this haplotype study, one and half 384-well plates are set up. This decision is due to the fixed scale of ABI assay products. Six 96-well plates are prepared for the sample preparation of one and half 384-well plates by Biomek 2000 robot. These wells contain six non-template controls (three for each plate) and three genomic controls (two for a 384-well plate and one for half 384-well plate). The concentration of samples in those 96 wells is evaluated by the RNaseP Assay and adjusted. First, the standard curve for DNA concentration was built using the serial dilution of 10 ng/μl ABI control DNA (Figure 10). The reaction efficiency was 96.4 % for the ABI DNA and 94.0 % for a sample DNA in this study.

Three SNPs in *ADRA2A* were selected based on their spacing and frequencies as described previously[143]. The promoter SNP, rs180044 (*Msp*I RFLP), was typed by a modified amplification of the region using deaza dGTP as described[109]. To summarize briefly, polymerase chain reaction (PCR) was performed in 20 μl reaction mixture containing 40 ng of genomic DNA, 62.5 μM each dATP, dTTP, dCTP, 31.25 μM dGTP, 31.25 μM deaza dGTP, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 unit of Taq DNA polymerase.

To test the valid concentration range for the Taqman assay, a test assay is conducted (Figure 10). The blue dots represent the serially diluted ABI control DNA. Most of them are well clustered as heterozygotes except the lowest concentration which is 0.35 ng for 5 ul reaction volumn. The scattered pattern follows highly dependent on the concentration, for example the highest concentration is located in the coordinate, 2.5 of X axis and 2.8 of Y axis. The arrow indicates .70 ng of our control DNA, which is lowest

concentration after serial dilution. All the other higher concentrations are well clustered as homozygote of allele X. The highest DNA amount was 45 ng, and all these are successfully clustered. Depending on the test result, the higher amount of DNA than 1.25 ng with Ct 26.5 is adjusted in setting up the plate for Taqman typing. The 2.25 μl (or 2.5 μl) of concentration-adjusted DNA in six 96-well plates were distributed to one and half 384-well plates by Biomek 2000 robot.

Figure 10. Standard curve using serial dilution of ABI control DNA.

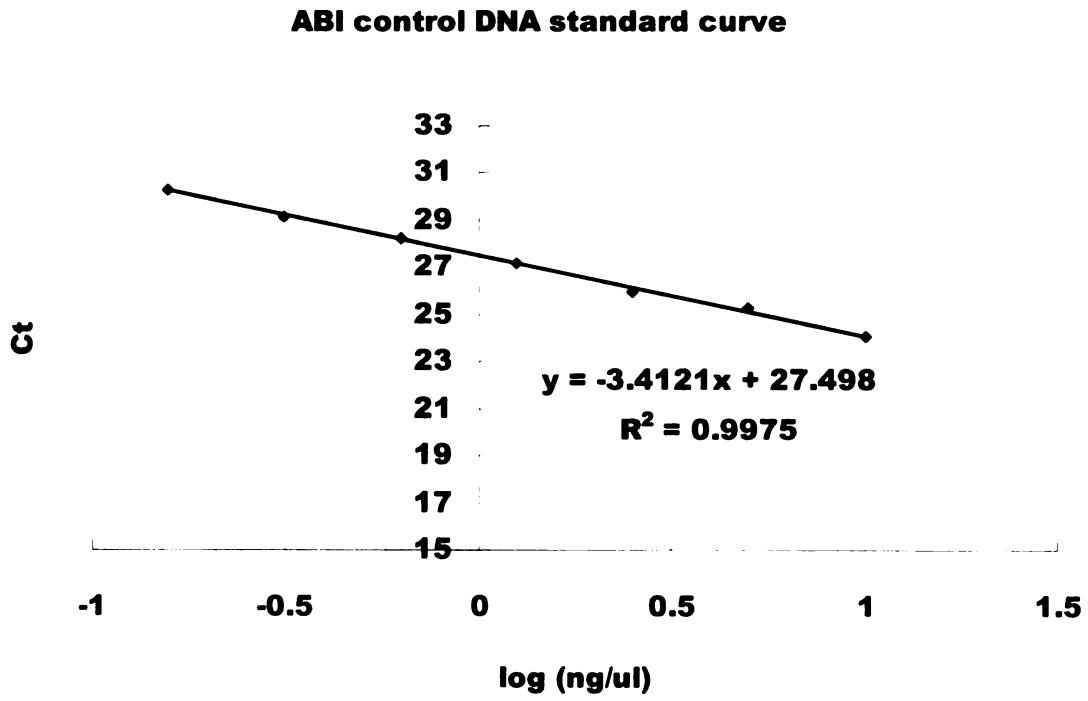
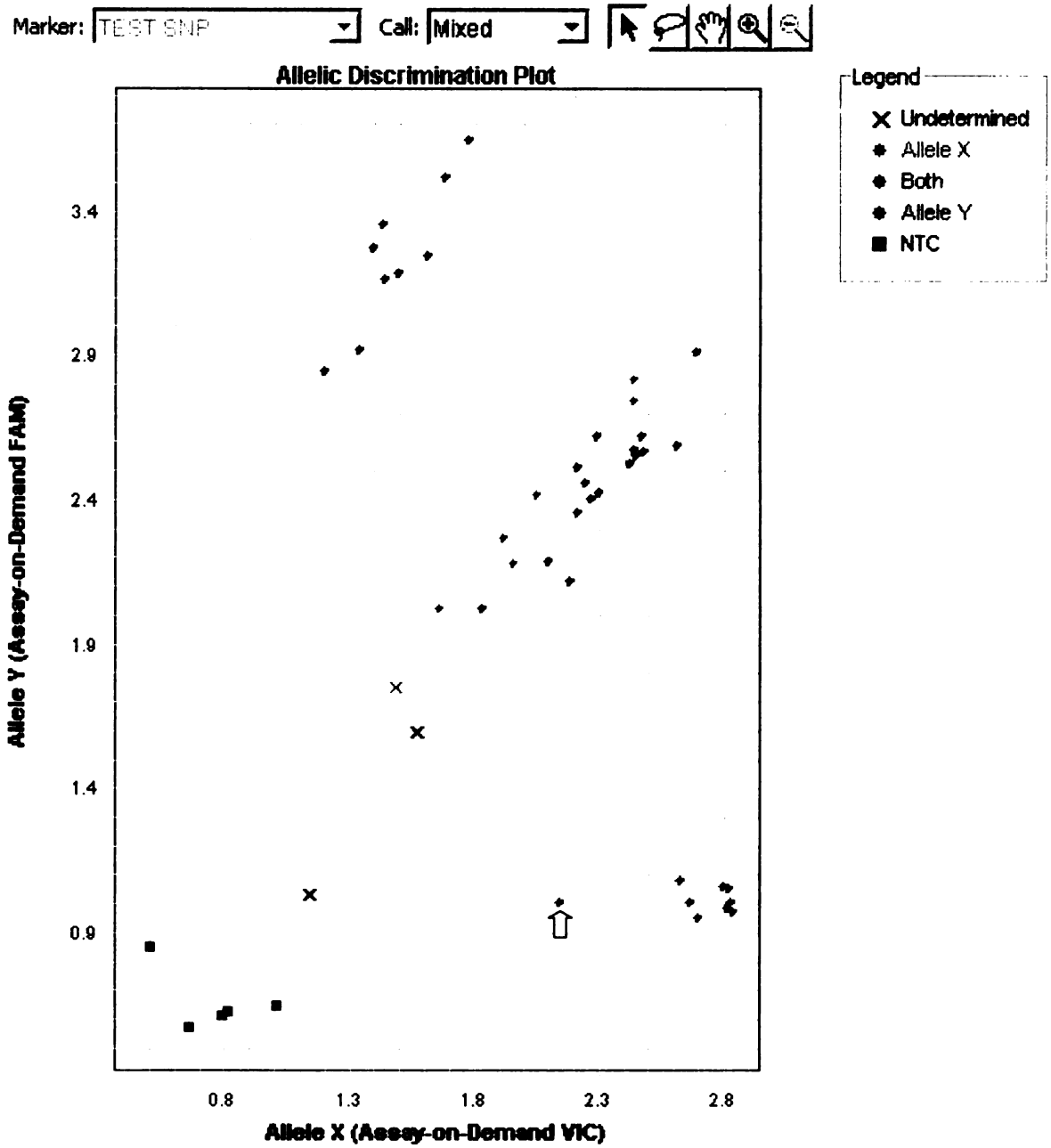


Figure 11. Allelic discrimination plot of a test assay.



In the case of limited samples, the whole genome is amplified using the Genomiphi DNA amplification kit and diluted for distribution to the 96 wells. To summarize, 1 µl of 20 ng/µl DNA is added to the sample buffer from Amersham biosciences. The mixture is heated for 3 minutes at 95 °C as a hot start and cooled down to 4 °C using the ABI 9700 PCR machine. The 9 µl of reaction buffer and 1 µl of the

enzyme mixture from the Amersham biosciences are added to the cooled mixture, and the mixture is incubated at 30 °C for 18 hours followed by the deactivation for 10 minutes at 65 °C. The amplified DNAs are diluted by adding 80 μl double-distilled water, and are stored in -20 °C until usage. The Genomiphi DNA amplification kit successfully amplifies around 10 μg of DNA from 20 ng DNA.

TaqMan assays

The procedure for the quantification as well as TaqMan genotyping is described below. The DNA targeted by the probes was amplified in 5 μl reaction mixture containing 1.4 ng or higher amount of DNA, 1X TaqMan universal PCR master mix with No AmpErase UNG and 1X Assay mix with the recommended thermal cycler condition consisting of two minute at 50 °C and an initial denaturing step for 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 92 °C (for quantification 95 °C) and one minute at 60 °C. The fluorescent probes cut during PCR were read by the ABI Prism 7900HT Sequence Detection System using the software, Sequence Detection System version 2.1. This procedure was mostly conducted at the Michigan State University Genomics Technology Support Facility.

Sequencing

The farther region of 3' UTR of *SLC6A3* containing SNPs, rs3797200 and rs18009939 was amplified by PCR in 20 μl reaction mixture containing 20 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer, and 0.5 units of Taq DNA polymerase. Amplification included an initial denaturing step at 94 °C for 3 minutes followed by 35 cycles consisting of 30 seconds at 94 °C, 30 seconds at 60 °C, and one minute at 72 °C, and the final extension step of 5 minutes at 72 °C with primer sets, 5'-GTGCGTGCCACATCAATAAC-3' and 5'-AACGAGACAAGGAGGC

TGAG-3'. The amount of amplified DNA was measured roughly by comparing to the standard marker, 100 bp DNA Ladder of New England Biolab (NEB) in 2% agarose gel stained with ethidium bromide. The 5 μl of amplified DNA was purified using 2 μl of UAB shrimp alkaline phosphatase (1 units/ μl) and 1 μl of USB exonuclease I (10 units/ μl). The reaction solution was mixed and incubated at 37 °C for 15 minutes followed by an inactivation at 80 °C for 15 minutes in a thermocycler. The sequencing was performed with at least 10-40 ng of purified DNA and 30 pmol of sequencing primer (the forward primer for PCR, 5'-GTGCGTGCCACATCAATAAC-3') at the Michigan State University Genomics Technology Support Facility using the ABI Prism 3700 DNA analyzer or ABI 3730 Genetic Analyzer.

Haplotype analysis

Case-control association tests with haplotypes were conducted using a coalescent-based Bayesian approach implanted in PHASE version 2.1[189,193]. PHASE can reconstruct haplotypes from a population constituted of unrelated individuals, and the case-control association test implemented in PHASE is a permutation-based likelihood ratio test. The default 100 permutations were performed in most results. If the p-value was .01 through 100 permutations, 1000 permutations were performed to get a right p-value.

For multilocus haplotypes from unphased genotyped data, pedigree transmission disequilibrium tests were performed using a generalized linear model for quantitative traits implemented in UNPHASED version 2.40[191]. The basic frame of this test is similar to the QTDT, but UNPHASED can handle multilocus haplotypes using EM algorithm. The results were permutated 1000 times to get the right p-values.

Chapter 5

Discussion

Introduction

The catecholamine pathway has been suspected as a main etiology for ADHD due to pathophysiology.[27] Among the genes related to the catecholamine pathway, two drug target genes, *ADRA2A* and *SLC6A3*, as well as *DRD4*, which has shown the most reliable association, were selected as candidate genes. The dopamine transporter gene, *SLC6A3*, is a target of methylphenidate, a stimulant drug. On the other hand, the α-2A-adrenergic receptor is a target of non-stimulant medication. As a result of this study, all three candidate genes, *ADRA2A*, *SLC6A3*, and *DRD4*, show associations with ADHD through several individual polymorphisms in each gene. Haplotype association studies also confirm the association between those genes and ADHD, although it is not clear which polymorphism is functional or how many functional polymorphisms are in each gene region without consistently higher detection using haplotype analyses.

This study reveals that the candidate gene association approach is sensitive in detecting the association of relevant genes. However, there are several intriguing findings. In the present chapter, these are discussed; as is the possible direction of future studies of complex traits.

LD and functional polymorphisms in haplotype analysis

In current types of haplotype analyses, since not all the polymorphisms in the gene region can be tested, there is always a possibility for type II error by missing functional polymorphisms, which reside in a gene region. A more elaborate approach is

necessary for preventing this possible error. One possibility is to theorize the LD between the functional polymorphisms and markers, and predict the detection power of association depending on the likelihood.

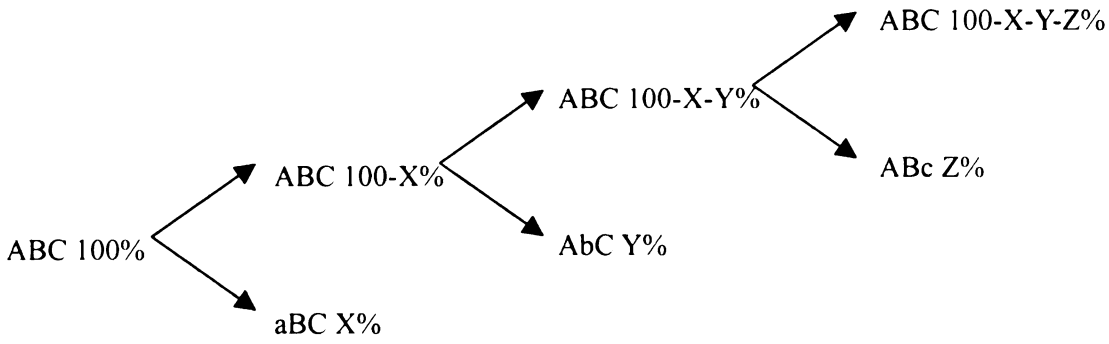


Figure 12. One possible description of haplotype structure using three SNPs.

Upper case: common allele; Lower case: rare allele; Most common SNP:A; Least common SNP: C.

In the previous chapter 2, the importance of polymorphism selection as it depends on two different LD measurements was discussed. The occurrence of two markers with high D' and low r^2 is probable when new polymorphisms are generated in a high LD block. As shown in Figure 13, if a new polymorphism emerged from a population, with no selection pressure on the SNPs, the haplotype harboring the new polymorphism, aBC, will be relatively rarer than the original haplotype, ABC. Therefore, the next polymorphism, AbC, probably also came from the original haplotype, ABC, too. If there are more ABCs than the other two haplotypes, aBC and AbC, the other polymorphism, ABc, has more chance to emerge from the original haplotype, ABC. However, depending on the presence of selection and bottle-neck effects, the more common haplotype could be different. Also, the chance appearance of a new SNP on a

rare haplotype could generate more possibilities. Considering all these possibilities but focused on only the final haplotype structures, the common haplotypes of a three-marker model are summarized in Table 34.

Table 34. Possible haplotypes of three-marker model depending on LD.

LD D'	LD r^2	Possible most common haplotypes*
>0.8	All>0.3	ABC>abc>aBC, abC
	A&B,C>0.3, B&C<0.1	ABC>abC>aBc>aBC
	A&B>0.3, C&A,B<0.1	ABC>abC>ABc>aBC
	A&C>0.3, B&A,C<0.1	ABC>aBc>AbC, aBC
	B&C>0.3, A&B,C<0.1	ABC>aBC>Abc>AbC
	All<0.1	ABC>aBC>AbC>ABc
0.4-0.8	All>0.1	ABC>abc>aBC, abC and others
	A&B,C>0.1, B&C<0.1	ABC>abC>aBc>aBC and others
	B&A,C>0.1, A&C<0.1	ABC>abC, aBC, Abc and others
	C&A,B>0.1, A&B<0.1	ABC>aBC, AbC>aBc, Abc and others
	A&B>0.1, C&A,B<0.1	ABC>abC>ABc>aBC and others
	A&C>0.1, B&A,C<0.1	ABC>aBc>AbC, aBC and others
	B&C>0.1, A&B,C<0.1	ABC>aBC>Abc>AbC and others
	All<0.1	ABC>aBC>AbC>ABc and others
<0.4	-	ABC>aBC, AbC, ABc, abC, aBc, Abc, abc

*Allele notation is the same as Figure 12.

Starting from the simplest case, there is one functional polymorphism, which has LD relationships with markers. If two markers are selected for detecting the functional polymorphism, the above three-marker model can be applied to predict the association detection. Likewise, marker model adding one more anonymous marker from the current selection of markers can be applied to the prediction for one functional polymorphism.

If there are two functional polymorphisms, the virtual examination using a two-marker model can be the first step; the construction of a whole-marker model using both functional polymorphisms and markers is the second step for detecting associations. Likewise, three or more functional polymorphisms can be calculated. The functional polymorphisms can be different in their influence on the trait. This can be incorporated easily if the amount of influence is calculable.

In general, the best detection of a functional polymorphism will involve those markers with simultaneously high D' and all low r^2 like the last case of D'>.8 in Table 34. However, in the case of constructing haplotypes with very rare markers, the detection of causative or protective haplotypes will be difficult due to the low relative frequencies of those haplotypes compared to the most frequent one. In other words, if three rare alleles with frequency of .1 are selected for construction of haplotypes, the maximum coverage has a probability of .7 within all possible haplotypes with all high D' and all low r^2 values. Therefore, primary allele frequencies for selection criteria would be in the range of .2-.3. If the LD blocks do not contain such polymorphisms with both high D' and low r^2 values, highly polymorphic markers, especially synonymous SNPs, can be selected instead to sort out the possibility of other haplotypes.

Differential detection of associations between TDT and case-control test

In this study, both the case-control association tests and TDTs were performed to find the association between the candidate genes and ADHD. However, as indicated previously, these two tests show inconsistency with each other in detecting associations. The gene *ADRA2A* reveals an association with ADHD mostly through TDT, whereas *DRD4* and *SLC6A3* show associations with ADHD mostly through case-control

association tests. In all three genes, separating the data according to the gender usually gives higher significance than total samples in either TDT or case-control association tests.

TDT is popular in light of the concern for population stratification in case-control studies. However, as mentioned earlier, if controls are well matched by ethnicity to affecteds or the frequencies of marker and disease alleles are not different among ethnic groups, population stratification is not a problem. The development of TDT starts from haplotype relative risk (HRR) using family-based controls instead of population-based controls. The important property of HRR regarding the independence of cells in contingency tables is indicated in recessive cases[194], and, from the observation that the linkage information is only stored in heterozygous parents in the contingency table, TDT is developed to test a linkage between a disease and marker loci in the presence of association[195]. However, it is unclear how TDT is applicable in dominant or additive cases. It is also noted that TDT and allele sharing statistics are mutually exclusive in testing a linkage in sib-pairs[195].

It is well recognized that TDT has much lower power with the same sample size due to examining the biased transmission from only heterozygous parents. Compared to HRR, which provides a valid test for the association using all parents, TDT tests a linkage from heterozygous parents in the presence of association. Missing informative transmission from homozygous parents may lead to a biased result for testing associations. It is notable that *ADRA2A* is associated with ADHD girls through the case-control association tests. Moreover, as mentioned in Chapter 2, the case-control tests between parents of controls versus parents of cases (ADHD-C) give significant *p*-values of .007 for In/ Del polymorphism in *DRD4* and .03 for VNTR in *SLC6A3*. Further

investigation is necessary for the explanation of differential detections between TDT and case-control association tests.

Opposite direction of VNTR on *SLC6A3* in our sample population

Although several association studies showed strong association between 3'UTR VNTR on *SLC6A3* and ADHD, the result was not consistent and finally meta-analysis could not detect significance[46]. More interestingly, the results in this study as well as another publication[35] find opposite significant association from other results. Also, several gene expression studies using 3'UTR VNTR showed inconsistency, as well (Table 35).

Table 35. *SLC6A3* expression assay depending on their VNTR genotypes.

Result	Method	Cell line	Gender	Tissue	Seq	Ref
10>9	Luciferase	COS-7	Parent:Male	Monkey Kidney	Avail	[180]
10>9	Quantitative RT-PCR	Brain samples	-	-	-	[196]
9>10	GFP	SN4741	Male	Mouse embryonic substantia nigra	-	[197]
9>10	Luciferase	SK-N-SH	Female	HS neuroblastoma Hyperdiploid	Avail	[179]
9>10	Luciferase	HEK-293	-	HS fetus hypotriploid	Avail	[178]
NS (9>10)	Luciferase	SN4741	Male	Mouse embryonic substantia nigra	-	[64]

NS: Not significant.

There are several studies related to the expression difference between genotypes. Most are performed using VNTR on the 3'UTR region. As shown in Table 31, the results

are not consistent depending on the genotypes. Interestingly, Table 32 shows that the dopamine transporter density using SPECT also replicated this inconsistency. A gene expression study using haplotypes on *SLC6A3* showed that promoter and intronic variants affect the transcriptional regulation of *SLC6A3* and suggested that particular combinations of polymorphisms in haplotypes affect the expression[64]. This result also may explain the reason why the dopamine transporter density is not consistent depending on the genotypes of a single polymorphism although it cannot explain the inconsistency of expression experiments of VNTR.

Table 36. SC6A3 density depending on their VNTR genotypes.

Result	Method	Disorder	Gender	Population	Region	Ref
*NS.**	SPECT [^{123}I]β-CIT	Schizophrenia		39 controls 29 patients	Striatum	198
10>9 *Total*	SPECT [^{123}I]β-CIT	Alcoholism	Avail	12 controls(4F, 8M) 17 patients(5F, 12M)	Striatum	199
9>10	SPECT [^{123}I]β-CIT	None	Avail	30 only controls (13F, 17M)	Striatum	200

NS: Not significant. *: But, amphetamine-induced decreased in [^{123}I]IBZM binding potential was *9>10 in each subgroups* (controls and schizophrenia).

SPECT results indicate that the dopamine transporter is concentrated in the striatum, but none of the gene expression studies are performed using cell lines derived from striatum. It may be possible that different cell lines express the dopamine transporter gene differentially. However, one study, the gene expression experiment using quantitative RT-PCR in brain and lymphocyte, suggests the possibility that it may not be necessary to look at the brain directly, if gene expression pattern is not different

depending on their genotypes in different individuals[196]. It seems that more delicate research is needed to find out the reason for inconsistency depending on the VNTR genotypes.

One SPECT study on the dopamine transporter found that the 10-repeat allele increases in dopamine transporter density[199], but another group found an opposite result using the same assay[200]. The other group reported no association between VNTR and dopamine transporter density[198]. For these SPECT studies, it is possible that there are many other polymorphisms affecting the gene transcription and translation. Because this gene is rather large (74,466 bp) and has two distinct block-like structures, it seems possible that combination of several different functional polymorphisms makes such a series of inconsistent results. Other polymorphisms in the gene region that affect expression previously are demonstrated already[64]. Although their result showed a bit more expression of the 9-repeat allele than the 10-repeat, it was not significant due to the existence of more significant polymorphisms via ANOVA.

Obviously, there are inconsistent results in gene expression studies shown in Table 31. One possible hypothesis is that sequence variability in VNTR may perform a real role in expression (Figure 13). Using the available sequence, a comparison was done between the sequences of VNTR and their expression assay results. Three results were possible to access the sequences[178-180]. As shown in Figure 14, four different sequence combinations were found from those studies.

Figure 13. VNTR subtypes.

A: AGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCAC

B: AGGAGC[A]TGTCCTATCCC[T]GGACGCATGCAGGGCCCCCAC

C: AGGAGCGTGT[A]CTA[C]CCC[A]GGACGCATGCAGGGCCCCCAC (most frequent)

D: AGGAGCGTGT[A]CTA[C]CCC[A]GGA[T]GCATGCAGGGCCCCCAC

E: AGGAGCGTGT[A]CTA[C]CCC[A]G[A]ACGCATGCAGGGCCCCCAC

F: AGGAGCGTGTCCTATCCCCGGAC[CGGAC]GCATGCAGGGCCCCCAC

G: AGGA[A]CGTGT[A]CTA[C]CCC[A]GGA[T]GCATGCAGGGCCCCCAC

H: [T]GGAGCGTGT[AT]TA[C]CCC[A]GGACGCATGCAGGGCCCCCAC

C': AGGAGCGTGT[A]CTA[C]CCC[A]GGACGCATGCAGGGCCCCCA[T]

C": [T]GGAGCGTGT[A]CTA[C]CCC[A]GGACGCATGCAGGGCCCCCAC

D': TGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCAC

Figure 14. *SLC6A3* expression depending on VNTR subtypes.

1) 9 repeat: AABECD'FDC' strongest

2) 10 repeat: AABECC"FCDC'

3) 9 repeat: AABECD'FCC'

4) 10 repeat: AABECHFCGC' weakest

This is one possibility to explain the inconsistency of associations. As previously shown, *SLC6A3* is associated with ADHD mostly in girls. It is also plausible that there may be some differential gene expression between gender groups depending on genotypes, although it should be carefully examined together with the morphological differences between genders and hormonal differences.

Possibility of genotyping error.

The importance of genotyping error has been recognized even with 1-2 % error rate[201,202]. Two kinds of errors are indicated; a pedigree error and a genotyping error[203]. The easiest detection of this error is to examine Mendelian inheritance of markers. Among a total of 228 families in this study, several families showed consistent non-Mendelian inheritance, probably due to one sample-mixed family and three non-parternity children (two of them have phenotypes). One child without the phenotype shows non-Mendelian markers twice and many failed genotypings suggesting that this DNA sample has poor quality. Those individuals are excluded from the data analyses.

Error detection based on the empirical penetrance model during genotyping is also suggested[204]. In case of a VNTR typing in this study, it is understood that the genotyped gel can be misread, even by an experienced laboratory technician. After this correction is once made, no further mistakes were seen, suggesting that proper training for genotypers can reduce genotyping errors. In the case of high-throughput genotypings using Taqman, the reading is done automatically through computer software. Due to the nature of this technology, it is far greater likely that the heterozygotes are true genotypes than homozygotes. Two such genotyping errors through Mendelian checks are suspected among ~9500 genotypes suggesting the observed error rate is approximately .0002 for the

TaqMan assays in this study. As indicated in the literature[205], there are further genotyping errors that are consistent with the Mendelian inheritance, and some of which can be found using additional error checking based on multipoint analyses in the case of cM-scale linkage studies.

Future studies

The importance of studying complex traits has been increasingly acknowledged, but outdated concepts and methodologies are still employed. In this transition period, it is important to recognize again the nature of differences between single gene disorders and polygenic complex traits. Without handling the polygenic nature in finding relevant genes, cures for complex disease will be still in a long way from right treatments.

The current linkage studies are moving from traditional Mendelian models to non-parametric affected sib-pair (ASP) methods. However, ASP methods do not basically consider the polygenic nature, and so lead to inconsistent results as summarized in Chapter 1. Apart from the whole genome scans, the candidate gene approaches are becoming popular due to the study design and many significant findings. This study reaffirms the sensitivity of candidate gene approaches by finding the associations between all three candidate genes and ADHD. An important note is this candidate gene approach also does not handle the polygenic nature of the disease. This flaw may lead to false positive or negative errors, or inconsistency among different studies, which are hard to explain.

This study gives three important messages: a) associations cannot be detected depending on a statistical approach, which means new approach or further explanation about differential statistical test results are necessary; b) there can be more than one

functional polymorphism in a gene region, which can be possibly protective as well as causative; c) a further study on gender difference on the target trait is necessary. Considering these findings, more elaborate approaches or new approaches are needed to find the real nature of complex traits.

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