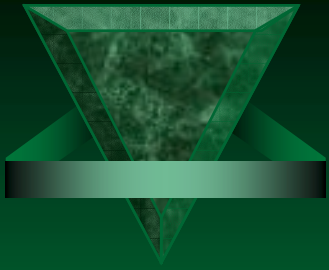


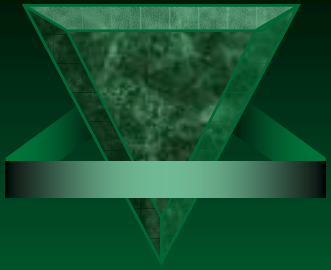
ADHD and DAT1: Affected Family-Based Control Study using TDT

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Outline

- Genetics in Medicine: overview
- Association Studies
- ADHD and DAT1: Affected Family-Based Control Study using TDT
- Data Analysis



Genetics in Medicine; Two Approaches

✔ Genetic Epidemiology

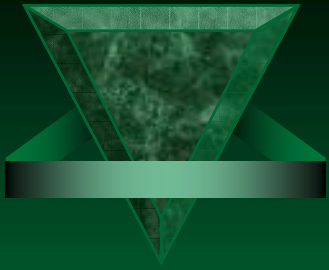
Macroscopic information (role of genetic and environmental factors, the familiarity of a disorder, and the mode of transmission)

✔ Molecular Genetics

Fundamental information (identification of the normal products of the vulnerability genes, when and where the genes are normally expressed in the developing CNS, and insights into how specific alleles function to establish disease vulnerability)

parametric methods - classical genetic linkage analysis

non-parametric methods - association studies, allele-sharing methods (affected sib-pair or affected relative studies)



Genetic Epidemiology (1)

- ✓ **Twin study (initial indicator of importance of genetic vs. non-genetic factors)**

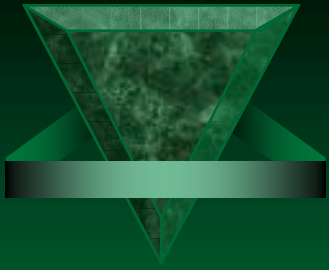
If MZ and DZ twins share their environment to the same extent, a higher concordance rate of disorders in MZ twins than in DZ twins suggests a strong genetic influence on the pathogenesis of the disorder

- ✓ **Adoption Study (Useful for distinguishing genetic and environmental influences confounded by the shared environment in family studies)**

1) a classic adoption study - compare the rates of disorders in biological relatives of the affected probands with those in the adoptive relatives

2) an adoptee's study to compare the rates of disorders in adopted offspring of affected parents with those in adopted offspring of unaffected parents

3) a cross-fostering study to compare the rates of disorders in adopted offspring of affected parents who were raised by unaffected parents with those in adopted offspring of unaffected parents who were raised by affected parents



Genetic Epidemiology (2)



Family Genetic Studies

- Investigate the rates and patterns of occurrence of disorders in biological relatives of probands with a disorder
- Useful tool for exploring the mode of transmission of a disorder
- Caution; information bias, possibility of cultural transmission (phenotypes of interest are transmitted within families by non-genetic mechanism)



Segregation Analysis

- Infer a best fitting mode of transmission of a disorder by ruling out those modes which do not fit the disorder
- Visual inspection of obvious segregation patterns in pedigrees and performing formal statistical testing

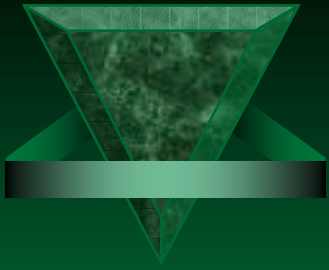


Molecular Genetics (1)

- ✓ Studies of cytogenetic abnormalities
 - Deletion and translocation of chromosomes
 - Clues to the chromosomal localization of disease vulnerability genes

- ✓ Parametric studies
 - Classical genetic linkage analysis

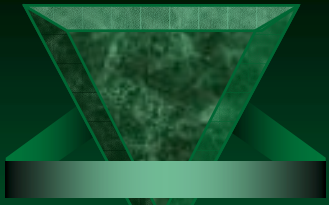
- ✓ Non-parametric studies
 - Association studies
 - Allele-sharing methods (affected sib-pair or affected relative studies)



Molecular Genetics (2)

Linkage Analysis (powerful tool due to its ability to locate disease vulnerability genes precisely)

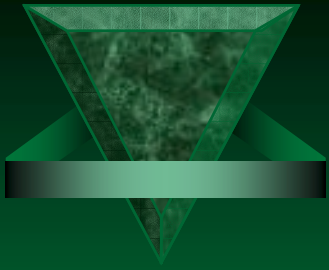
- ✓ Attempt to identify disease vulnerability genes by investigating the association between the transmission pattern of a disorder in the pedigree and the linkage between a known genetic marker and putative genes thought to be responsible for disease, by detecting an RF smaller than 0.5 and estimating the magnitude of the linkage.
- ✓ A limitation of linkage analysis
 - 1) disease model dependent (specifically, in psychiatric disorders, depends on correct assumptions regarding the involvement of a single major gene in the disease transmission, genetic homogeneity, and the precise mode of transmission)
 - 2) statistical multiple testing
 - 3) limited applicability in disorders with complex traits, such as phenotypic variation, phenocopies, incomplete penetrance, genetic heterogeneity, polygenic inheritance and a high frequency of disease causing alleles in the population.



Molecular Genetics (4)

Association studies

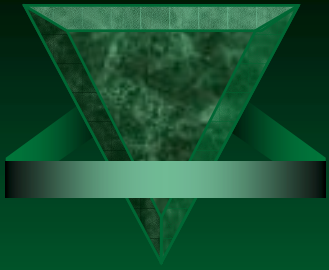
- ✔ Detect a difference in allele frequencies at a particular locus, using a case-control study design.
- ✔ A positive allele association can indicate
 - 1) the marker examined plays a causative role in the disorder
 - 2) the marker is in linkage disequilibrium, and therefore, has no direct effect on the pathogenesis of the disorder but is closely linked to a disease-causing gene
 - 3) it is a false positive finding due to either an artifact of population admixture (ethnic difference in allele frequencies) or polymorphism in the marker which leads to consequent multiple testing.
- ✔ Amendment for false positive findings
 - 1) using a homogeneous population or using an internal comparison group, (affected family-based control)
 - 2) Statistical adjustment for multiple testing
- ✔ The advantages of association studies
 - lack of a requirement for transmission models or assumptions and their potential to detect genes of small effect.



Molecular Genetics (5)

A candidate gene approach

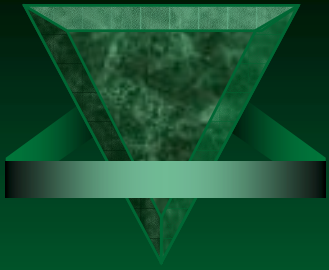
- A special case of marker disease association study with a recombination fraction of 0 between marker and disease locus
- Candidate genes that are implicated in the pathogenesis of the disorder (e.g.; genes coding for specific NTs or receptors)



Molecular Genetics (6)

The allele-sharing methods

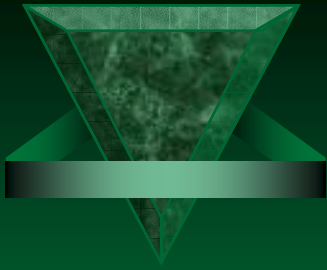
- Affected sib-pair analysis, affected relatives analysis of a pedigree
- Detect disease vulnerability genes: non-parametric method to detect linkage
- Investigators examine whether the inheritance pattern of a chromosomal region is inconsistent with random Mendelian segregation by showing that affected relatives or siblings inherit identical copies of the region more often than expected by chance



Association Studies (1)

Linkage Equilibrium

- ✓ Specific alleles at 2 loci; M, D
- ✓ M & D is in linkage equilibrium = $P [MD] \text{ in gamete} = P[M] * P[D]$
- ✓ Deviation from this independent presence of M and D in haplotypes -> allelic association or linkage disequilibrium
- ✓ Recombination reduce the linkage disequilibrium by a factor of $(1-\Theta)^n$ (Θ ; recombination fraction – probability of a recombination between 2 loci, n; generation)
-> linkage disequilibrium can persist for hundreds of generations in tightly linked loci

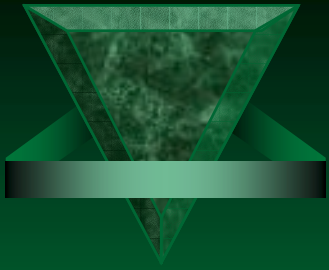


Association Studies (2)

- ✓ Example; Ankylosing spondylitis (recessive gene)

	At least 1 Allele at a marker loci		
	B27(+)	B27(-)	Total
Disease D(+)	72	3	75
Status D(-)	3	72	75
Total	75	75	150

- ✓ RR (relative risk)
 - = $P[\text{develop disease with risk factor}] / P[\text{develop disease without risk factor}]$
 - = OR (in rare diseases)
 - = $72 \cdot 72 / (3 \cdot 3) = 576$

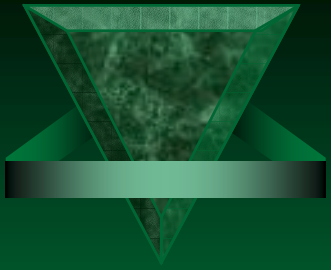


Association Studies (3)

✓ Caution on interpretation

Association can arise as an artifact due to population admixture

(e.g.) association study between trait of the ability to eat with chopsticks and the HLA-A locus in the SF, then allele A1 would be positively associated because both the ability to use chopsticks and allele A1 is more frequent among Asians than Caucasians



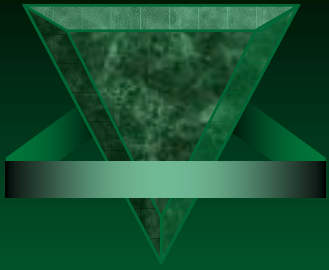
Affected Family-based Control Association Studies (1)

- ✓ Basic Ideas (Rubinstein et al., 1981; Falk & Rubinstein, 1987)

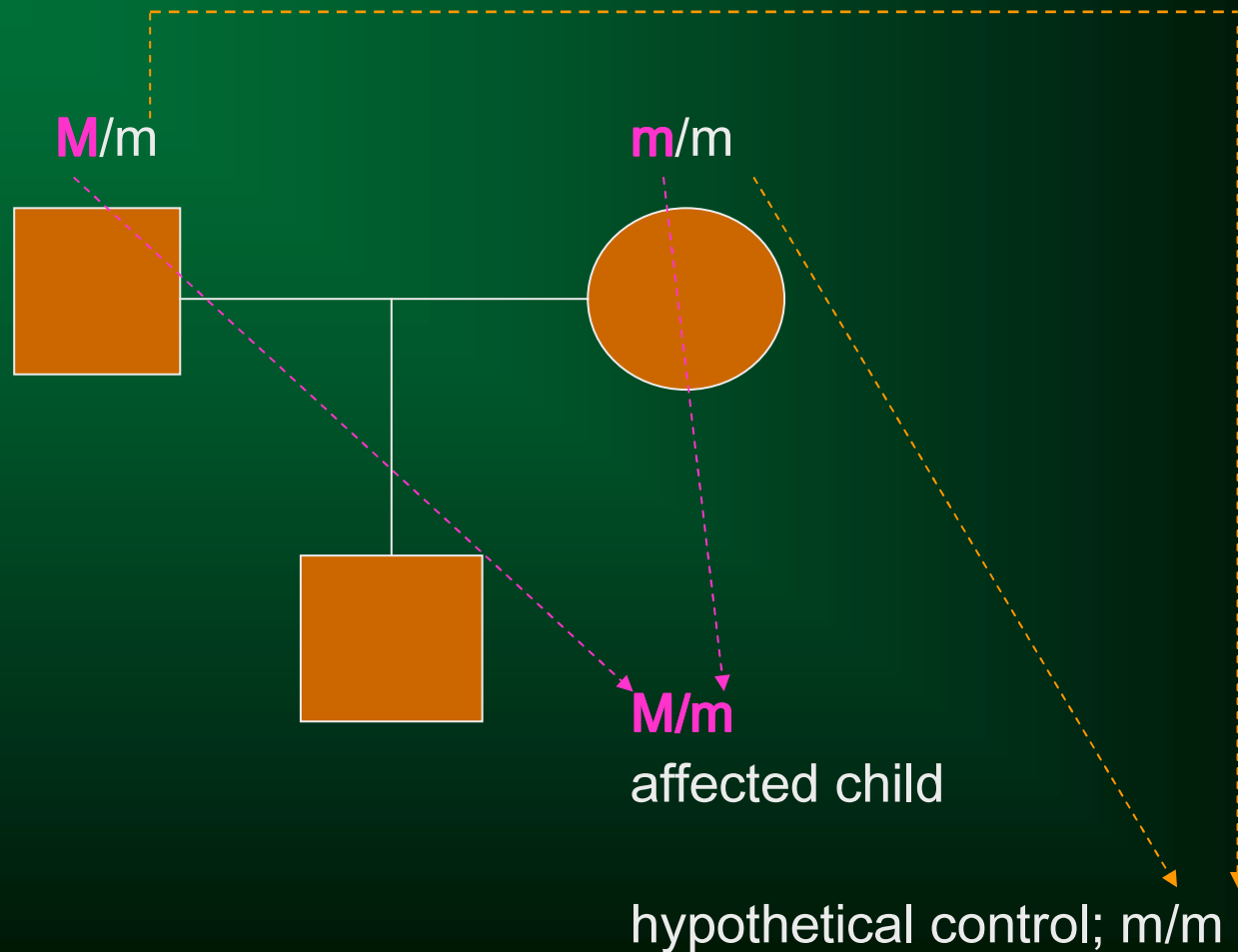
To avoid the difficulties in selecting appropriate control group, use parental data in place of non-related controls

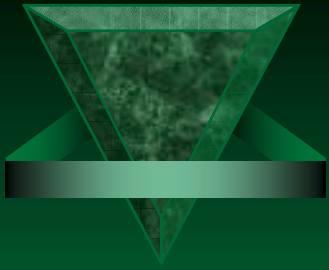
- ✓ Subjects

Nuclear family with a single affected child -> type at the marker locus -> 2 parental alleles not transmitted to the affected child and they serve as a hypothetical control individual



Marker Genotypes in Nuclear Family (concept for AFBAC)





Affected Family-based Control Association Studies (2)

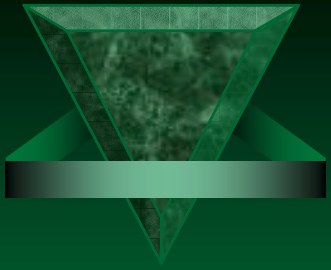
Comparison with association studies

✦ Disadvantages

- 1) more genotyping (2 in association studies, 3 in affected family-based control studies)
- 2) Difficulty in sampling of trios

✦ Advantages

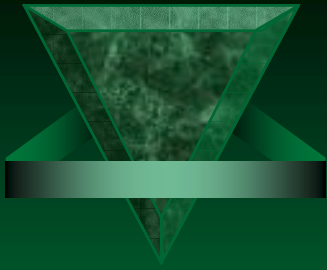
overcoming problem of population stratification and false positive results of case-control studies



Affected Family-based Control Association Studies (3)

Case-Control Study

	Genotype		Total
	M (+)	M (-)	
Case	23	6	29
Control	14	15	29
Total	37	21	58



AFBAC1

Haplotype Relative Risk (HRR) (1)

- Genotype-based Analysis (heterozygosity of allele M is not important) ; recessive model

		Non-Transmitted genotype		Total
		M (+) M/M, M/m	M (-) m/m	
Transmitted Genotype	M(+)	9	14	23 (W)
	M(-)	5	1	6 (X)
Total		14 (Y)	15 (Z)	29 (n)

- each family contribute to one cell (total of 29 families)
- Assumption; distribution of marker genotypes from NT parental alleles in families is identical to the distribution of marker genotypes in the population.

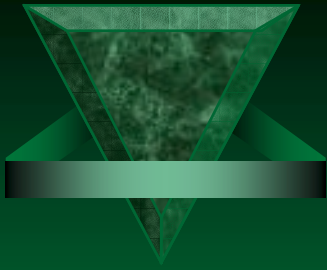


AFBAC1

Haplotype Relative Risk (HRR) (2)

- ✓ HRR =
$$\frac{W \text{ (M (+) affected child frequency)} / X \text{ (M (-) affected child frequency)}}{Y \text{ (M (+) in NT parental alleles)} / Z \text{ (M (-) in NT parental alleles)}}$$

$$= (W*Z) / (X*Y) = (23*15) / (6*14) = 4.17$$
- ✓ H_0 ; No association
Test statistics; χ^2 test $(14-5)^2 / (14+5)$, $df=1$, $p=0.039$
- ✓ Similarity of HRR with RR in case-control studies
- ✓ $\theta = 0$, HRR=RR (no recombination = linkage)

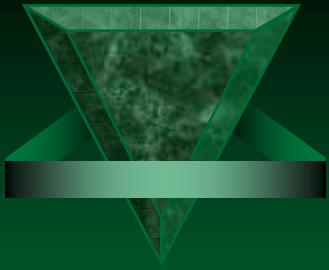


AFBAC2

Haplotype-Based Haplotype Relative Risk (HHRR) (1)

- ✓ Terlinger & Ott (1992)
- ✓ Compares observed frequencies of allele M in T (case) and NT (control) alleles (each parent has 2 allele, each family has 2 parents, thus total is 4n); unmatched analysis of TDT

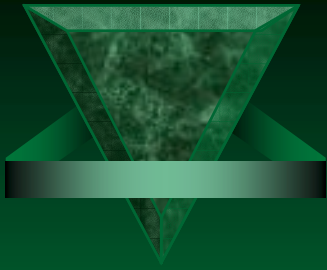
	Parental allele		Total
	M	m	
Transmitted	52	6	58
Non-transmitted	39	19	58
Total	91	25	116



AFBAC2

Haplotype-Based Haplotype Relative Risk (HHRR) (2)

- ✓ Assumption;
 - 1) contribution of the parents are independent
 - 2) T and NT genotypes are independent
- ✓ H_0 ; no association
- ✓ Test statistics; χ^2 test



AFBAC3

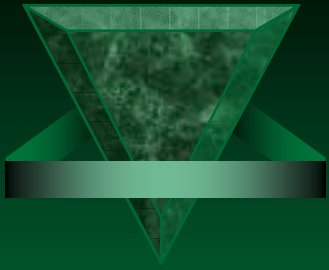
Transmission/Disequilibrium Test (TDT) (1)

- ✓ Spielman et al. (1993)

		Non-Transmitted allele		Total
		M	m	
Transmitted	M	33	19	52
	m	6	0	6

Total		39	19	58

- ✓ Classify each single parent according to his/her T and NT allele (Terwilliger & Ott; 1992)
- ✓ Each parent count per family $\rightarrow 2n$ ($29 \cdot 2 = 58$); 58 parents in 29 families



AFBAC3

Transmission/Disequilibrium Test (TDT) (2)

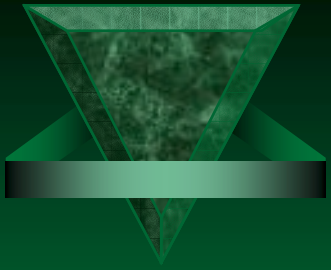
- ✓ H_0 ; no association and linkage
 $\delta(1-2\Theta) = 0$
($\delta=0$; each heterozygous parent transmits its M allele to the affected child with probability of $\frac{1}{2}$ -> no association
 $\Theta=1/2$; no linkage)
- ✓ Test statistic;
McNemar's χ^2 test
 $= (b-c)^2/(b+c) = (19-6)^2/(19+6) = 6.76$ ($p=0.0093$)
Only heterozygous parents contribute to the analysis
- ✓ Limitation
TDT can detect linkage between the marker locus and the disease locus only if association (due to linkage disequilibrium) is present



DAT1 and ADHD: Affected Family-Based Control Study using TDT

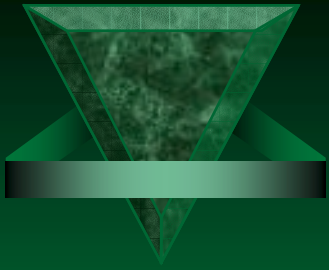


- ✓ Characteristics of Attention Deficit- Hyperactivity Disorder (ADHD)
 - Attention problem (inattention, distractibility)
 - Hyperactivity, impulsivity
- ✓ Common; 3-6%
- ✓ Risk factor for antisocial and drug abuse in adulthood



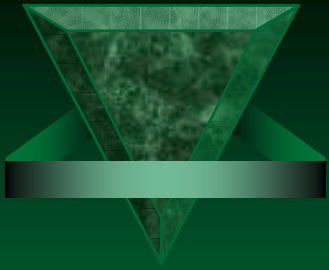
Heritability of ADHD

- ✓ Twin studies
 - ≥ 0.8 heritability estimates
- ✓ Family studies
 - more ADHD in relatives of probands with ADHD compared to relatives of adoptive parents, normal controls, or psychiatric controls
- ✓ Segregation analysis
 - (1) autosomal dominant transmission with reduced penetrance of the hypothesized gene
 - (2) single-gene effect vs. polygenic inheritance



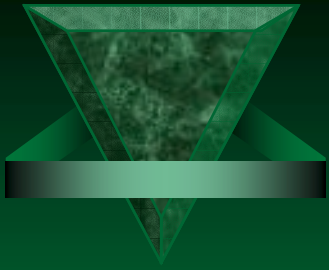
DAT1 as a Candidate Gene

- ✓ DAT1
 - VNTR (variable numbers of tandem repeats) of a 40 base-pair repeat sequence on chromosome 15.3
 - Majority; 10 repeats or 9 repeats
- ✓ DAT1 and ADHD
 - Dopamine hypothesis; children with ADHD responds well to dopamine agonists and has inhibitory effects on the dopamine transporter (DAT1)
 - Animal studies
 - 1) overexpression of mutant rat dopamine transporter
 - 2) mucous 'knockout' studies



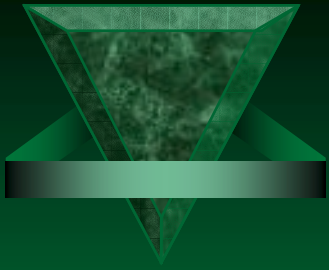
Aim of the Study

- To test the previously found family-based association of ADHD with the dopamine transporter 10-copy (480 bp) allele in this larger Korean sample



Study Subjects

- ✓ Challenge; reduce phenocopy (someone plays the behavioral symptoms of ADHD without hypothesized genetics etiology)
- ✓ Inclusion criteria
 - (a) Diagnosis of DSM-IV ADHD, Combined Type, determined by concurrence of two independent diagnosticians after review of all clinical information, corroborated by K-SADS and combined parent and teacher reports
 - (b) Age between 6 and 12
 - (c) WISC-III Full Scale IQ > 80
 - (d) Participation of both biological parents
 - (e) Parent informed consent and child assent
- ✓ Exclusion criteria
 - (a) Seizure disorder or neurological disease, bipolar mood disorder, pervasive developmental disorder, Tourette syndrome or chronic motor tic disorder, or uncorrected sensory impairment.
 - (b) Parental history of bipolar mood disorder



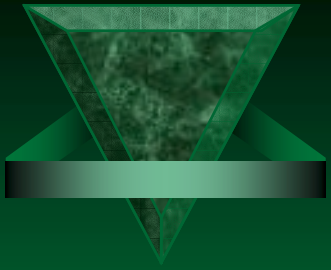
Genetic Lab Procedures

✓ Dopamine transporter genotyping

1) PCR will be carried out in a 10 l volume containing 50 ng of genomic template, 0.5 M of each primer, one of which is 5' fluorescently labeled, 200 M of each dNTP (dATP, dCTP, dGTP, dTTP), 1 x PCR buffer, 2 mM MgCl₂, and 0.5 units Taq polymerase (Amplitaq Gold). Samples will be amplified on a 9700 thermal cycler with an initial 12 minute step to heat-activate the enzyme, 40 cycles consisting of a denaturation step of 95 degrees C for 30 sec., an annealing step of 68 degrees C for 30 sec., and an extension step of 72 degrees C for 30 sec.


2) Products will be injected on an ABI 3700 multi-capillary array genetic analyzer with POP6 polymer. Products will be detected by laser-induced fluorescence using sheath flow on the ABI 3700.

Electropherograms will be processed with Genescan software and alleles will be called with Genotyper software, blind to all but a number which is consecutively assigned and is not related to whether the subject is a child, father, or mother and without any indication of relationship to adjacent numbers.

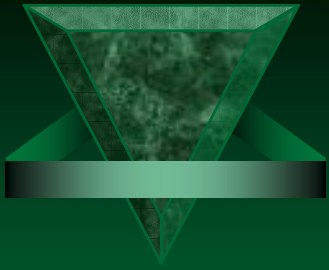


Preliminary Analysis

- ✓ 25 complete trios with ADHD combined type.
- ✓ Novel allele found;
365bp (7 repeat allele)
- ✓ Linkage Format



Family ID	Subject ID	Dad ID	Mom ID	Gender	Affected State	Allele 01	Allele 02	483T	483NT
K024	462	0	0	1	1	444	483		1
K024	272	0	0	2	1	483	483		
K024	439	462	272	1	2	444	483		
K025	458	0	0	1	1	483	521	1	
K025	278	0	0	2	1	444	483	1	
K025	444	458	278	1	2	483	483		
K026	285	0	0	1	1	444	483		1
K026	494	0	0	2	1	483	483		
K025	449	285	494	1	2	444	483		



2X2 Table Illustration (1)

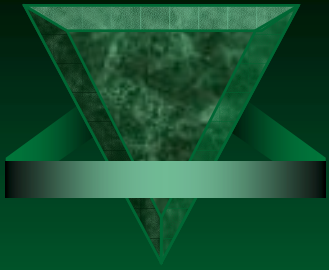
▼ HRR Analysis

		Non-Transmitted genotype		
		483 (+)	483 (-)	Total
Transmitted Genotype	483 (+)	24	1	25 (W)
	483 (-)	0	0	0 (X)
Total		24 (Y)	1 (Z)	25 (n)

$$\text{HRR} = (25 * 1) / (0 * 1) = \infty$$

χ^2 test = not calculable due to 0s in cells

Accept null hypothesis; no association



2X2 Table Illustration (2)

✓ HHRR Analysis

		Parental allele		Total
		483(+)	483(-)	
Transmitted		43	7	50
Non-transmitted	46	4	4	50
Total		89	11	100

χ^2 test = 0.919 (p=0.338)

Accept null hypothesis; no association



2X2 Table Illustration (3)

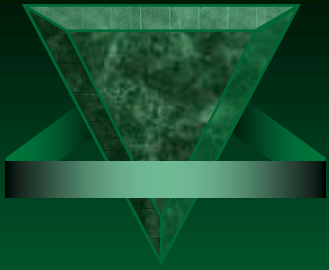
▼ TDT Analysis

		Non-Transmitted allele		Total

		483 (+)	483 (-)	
Transmitted allele	483 (+)	39	4	43
	483 (-)	7	0	7

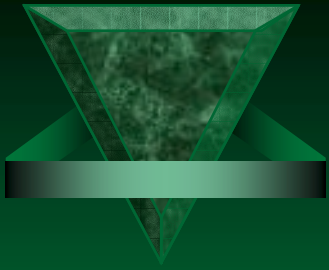
Total		46	4	50

McNemar's χ^2 test with 1 d.f. = $(4-7)^2/(4+7) = 0.8182$. ($p \gg 0.05$)
Accept null hypothesis; no association and no linkage



Implications

- ✓ Possible that there is no association and linkage in Korean children with DAT1; difference with Caucasian children with ADHD
- ✓ Limitations
Small sample size and small number of heterozygous allele parents provided limited information on the TDT analysis
- ✓ Analysis of more samples (aimed at least 120 trios) are underway



Acknowledgment

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