

A High Density SNP Linkage Scan with 142 Combined Subtype ADHD Sib Pairs

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Abstract

As part of the International Multi-centre ADHD Gene (IMAGE) project we have completed an affected sibling pair study of 142 narrowly defined DSM-IV combined type ADHD proband-sibling pairs. We found suggestive linkage on chromosomes 9 and 16 with non-parametric multipoint peak LOD scores of 2.13 and 3.1 respectively. There have been several previous ADHD linkage scans. The UCLA study (Fisher et al. 2002; Ogdie et al. 2004; Ogdie et al. 2003), the Dutch study (Bakker et al. 2003), the German study (Hebebrand et al. 2006) and the MGH Study (Faraone et al., submitted) applied the affected sib pair (ASP) strategy; the Columbian study used extended pedigrees ascertained from a population isolate (Arcos-Burgos et al. 2004). No linkage was observed on the most established ADHD linked genomic regions from these studies on 5p and 17p. The highest multipoint nonparametric linkage signal on chromosome 16q23 at 99 cM (LOD=3.1) overlapped with data published from UCLA (LOD>1, ~95cM) and Holland (LOD>1, ~100cM). The second highest peak in this study was on chromosome 9q22 at 90cM (LOD=2.13). Both the UCLA study and the German study also found some evidence of linkage at almost the same location (UCLA LOD=1.45 at 93cM; German LOD=0.68 at 100cM). The overlap of the two main peaks from this study with those from previous studies suggests that true loci linked to ADHD may lie within these regions.

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is one of the most common behavioral disorders of childhood, characterized by the early onset of age inappropriate hyperactivity, impulsivity and inattentiveness. Family studies indicate a sibling relative risk (λ_s) around 4 to 6 (Brookes et al. 2006; Faraone et al. 2000; Smalley 1997). A recent review of 20 population twin studies from around the world found an average heritability of 76% suggesting that genetic influences play a major role in the etiology of ADHD (Faraone et al. 2005). Candidate gene studies have so far confirmed associations to genetic variants within or close to the dopamine D4 and D5 receptor genes (Li et al. 2006) and identified variants in several other genes that appear to increase the risk of ADHD (Faraone et al. 2005). The genetic variants identified so far contribute only a small amount to the phenotypic variance explained by genetic

factors and the expectation is that further genes will be found that are associated with the disorder (Kuntsi et al. 2006).

A major strategy in the search for novel genes that increase the risk for ADHD and other complex disorders is the use of genome-wide linkage scans to identify chromosomal regions containing susceptibility loci. To date five independent ADHD genome-wide linkage scans have been completed. Four of these studies, from North America (UCLA) (Fisher et al. 2002; Ogdie et al. 2003) and MGH (Faraone et al., submitted), the Netherlands (Bakker et al. 2003) and Germany (Hebebrand et al. 2006) applied an affected sib pair (ASP) strategy, while the fifth used extended pedigrees ascertained from a population isolate in Columbia (Arcos-Burgos et al. 2004). With the exception of the MGH scan, each study identified novel genomic regions

that might harbor ADHD associated genes, although as yet no genes explaining any of the linkage findings have been reported in the published literature. The most significant findings were reported on chromosomes 5p and 17p where linkage signals were found in multiple studies (Arcos-Burgos et al. 2004; Hebebrand et al. 2006; Ogdie et al. 2004). However, none of these loci were consistently found, no study achieved genome-wide significance and most observations were not replicated. This indicates the complexity of ADHD genetic etiology and is largely in line with findings from other common complex genetic disorders. Moreover, linkage studies are poorly powered to detect genes of small effect (Risch 1990) and the use of modest sample sizes is therefore expected to lead to a high level of fluctuation in the detection of true linkage signals.

In the current study we contribute to linkage findings from affected sibling pair studies of ADHD, using a sample of 142 DSM-IV combined type ADHD proband-sibling pairs, ascertained by the International Multi-centre ADHD Gene (IMAGE) project (Brookes et al. 2006; Kuntsi et al. 2006). Using a panel of highly informative SNPs our main aim was to identify linkage regions using a sample ascertained with a relatively homogeneous phenotype. Latent class analysis suggests that the DSM-IV combined subtype of ADHD falls entirely within an empirically derived latent class that shows a high level of subtype concordance within twin pairs (Rasmussen et al. 2004; Todd et al. 2001). Furthermore, combined subtype ADHD is the most common presentation of ADHD within child and adolescent psychiatric

clinics and is associated with high levels of hyperactive-impulsive, inattentive and comorbid problems (Faraone et al. 1998). In light of the genetic heterogeneity observed within our multi-country sample (Neale et al., submitted), we also performed simulations to investigate its impact of this heterogeneity on the linkage signals observed in this study.

MATERIALS AND METHODS

Sample Ascertainment and Diagnosis

Families were identified by the IMAGE consortium through clinically diagnosed combined subtype ADHD cases at specialist clinics in 8 European countries: Belgium, England, Germany, Ireland, Israel, Netherlands, Spain and Switzerland. To ensure the genetic homogeneity of the sample only white Caucasians of European ancestry were included based on information on ethnicity going back to grandparents of the affected sibling pairs. Entry criteria for research assessments were: (1) a proband with the ADHD combined subtype, (2) availability of one or more siblings of each affected proband, (3) both the probands and siblings aged between 5 and 17 at the time of assessment, (4) both the children and at least one of their biological parents were available for DNA collection. Eligible families were then invited to the research centers or assessed during home assessments by qualified child psychiatrists or trained interviewers using clinical research assessments. Blood or buccal samples were collected for DNA extraction. Exclusion criteria applied to both probands and siblings: (1) IQ<70, (2) a diagnosis of schizophrenia or autism that might confound the diagnosis of

ADHD, (3) neurological disorders such as epilepsy and brain injury, as well as any genetic or medical disorder associated with externalizing behaviors that might mimic ADHD.

Clinical Procedures

Parental Account of Childhood Symptom (PACS):

PACS is a semi-structured, standardized, investigator-based interview developed as an instrument to provide an objective measure of child behavior (Taylor et al. 1986a; Taylor et al. 1987; Taylor et al. 1986b). A trained interviewer administers PACS with parents, who are asked for detailed descriptions of the child's typical behavior in a range of specified situations. Such situations are defined either by external events (e.g. watching television, reading a book or comic, playing alone, playing with friends, going to bed, traveling) or by behaviors shown (e.g. crying, worried talk, tempers, fighting with siblings). Interviewers then make their own ratings on the basis of a formal training and written definitions of the behaviors to be rated, on a 4-point scale of severity and frequency in the previous week and previous year. Interrater reliability is high with product-moment correlations for pairs of interviewers ranging from 0.92-0.95 for 'hyperactivity', 0.89-0.95 for 'defiance' and 0.79-0.90 for 'emotional problems' (Taylor et al. 1986b). The internal consistency of the scales for behavior was acceptably good: Cronbach's alpha was 0.89 for 'hyperactivity' and 0.87 for 'defiance' (Taylor et al. 1986b). The Hyperactivity subscale is made up of attention span (time spent on a single activity, rated separately for four different kinds of activity), restlessness (moving about during the same activities), fidgetiness (movements of parts of the body during the same activities), and

activity level (rated for structured situations such as mealtimes and car journeys), with other subscales covering defiant, emotional and other comorbid disorders including autistic spectrum disorders.

Rating Scales: Rating scales used to quantify ADHD symptoms included the Long Version of Conners' Parent Rating Scale (CPRS-R:L), the Long Version of Conners' Teacher Rating Scale (CTRS-R:L) and parent and teacher versions of the Strengths and Difficulties Questionnaires (SDQ) (Conners 1995; Conners 2003; Goodman 1997). In the analyses presented here only ADHD symptoms derived from the CTRS-R:L were included as part of the diagnostic algorithm for ADHD (see below). In order to exclude autism spectrum disorders that might confound the analysis of ADHD, both probands and siblings were screened using the Social Communication Questionnaire (Rutter et al. 2003) in conjunction with the pro-social scale from the SDQ. Individuals falling above thresholds for the SCQ (≥ 15) and SDQ (≤ 4) were further evaluated using the autism spectrum disorder section of the PACS interview. A diagnosis of possible autism or autism spectrum disorder led to exclusion from the study.

DSM-IV Diagnoses: All raw data was centralized and stored on a secure database at the MRC Social Genetic Developmental Psychiatry research centre in London. A standardized algorithm was applied to PACS to derive each of the 18 DSM-IV ADHD items, providing operational definitions for each behavioral symptom. These were combined with items that scored 2 or 3 from the teacher rated Conners' ADHD

subscale, to generate the total number of items from the DSM-IV symptom checklist. Situational pervasiveness was defined as some symptoms occurring within two or more different situations from the PACS interview, or the presence of one or more symptoms scoring 2 or more from the ADHD subscale of the teacher rated Conners'.

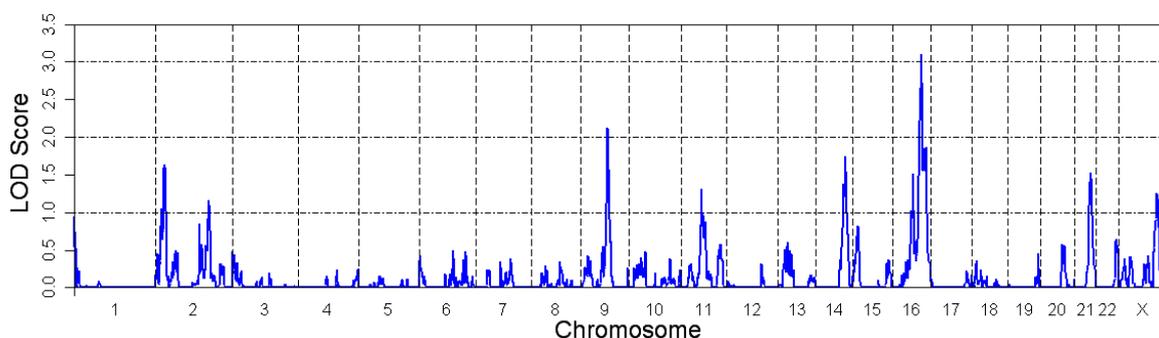
Sample selection: After pedigree correction using the linkage markers to check for expected family relationships, the final sample in this study consisted of 577 subjects, including 276 ADHD combined subtype cases from 134 nuclear families. With 8 families having 3 affected children, a total of 142 independent sibling pairs with combined subtype ADHD were formed. Table 1 summarizes the

clinical characteristics of the sample according to the ascertainment procedure, alongside those from previously published affected sibling pair studies of ADHD. Genotypes were available to maximize identity-by-descent (IBD) information from both parents in 111 families and from one parent plus an additional unaffected sibling in the remaining 23 families. All the families gave informed consents for interview, DNA and cell line storage at Rutgers University Cell and DNA Repository (RUCDR; Piscataway, New Jersey, USA) and sharing of anonymous clinical and genotype data at the NIMH central depository. Ethical approval for the study was obtained from National Institute of Health registered ethical review boards from each centre.

Table 1 Clinical features of the ADHD children from the IMAGE project and other affected sibling pair studies of ADHD: UCLA (Fisher et al. 2002; Ogdie et al. 2003), Netherlands (Bakker et al. 2003), Germany (Hebebrand et al. 2006).

Study	IMAGE		UCLA		Netherlands		Germany	
Number of affected siblings	276		438		199		229	
% Male	78.6		71.9		83.9		72.1	
% White Caucasian	100%		80%		100%		100%	
	N	%	N	%	N	%	N	%
ADHD Subtype								
Combined	276	100	211	48	170	85	170	85
Inattentive	0	0	196	45	25	13	25	13
Hyperactive-impulsive	0	0	31	7	4	2	4	2
Co-morbid disorder								
Oppositional defiant disorder	156	0.57	82	55	55	24	82	41
Conduct disorder	72	0.26	14	8	8	3	14	7
Mood disorder	41	0.15	15	4	4	2	15	8
Anxiety disorder	96	0.35	27	18	18	8	27	14
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age	10.6	3.10	10		10	3	10	3.00
IQ	98.3	14.50	> 80		> 80	-	> 80	-

Figure 1 Nonparametric multipoint LOD scores for ADHD-CT diagnosis



Genotyping and Data Cleaning: The genotyping service was provided by the Center for Inherited Disease Research (CIDR; <http://www.cidr.jhmi.edu/>) using the Illumina BeadArray™ technology on a BeadLab system. A total of 5,873 SNPs from the Illumina Linkage IVB SNP panel were successfully assayed with a reproduction rate of 99.994%. The markers were ordered and placed on the physical map according to Genome Build 35. Interpolated genetic distances from the deCODE genetic map were used to estimate genetic map distances (Kong et al. 2002).

Pedigree errors were identified and corrected by testing pair-wise subject relationships with the program RELPAIR (Epstein et al. 2000). Genotypes causing Mendelian inconsistencies were identified by PEDCHECK and removed by a custom script (O'Connell and Weeks 1998). Hardy-Weinberg Equilibrium (HWE) for each marker was calculated using. 138 SNP markers were dropped that showed significant departure from HWE ($p \leq 0.01$). Unlikely combinations leading to double recombinations over short genetic distance in some families were also removed by MERLIN (Abecasis et al. 2002; Wigginton and Abecasis 2005). 138 SNP markers were dropped that showed

significant departure from HWE ($p \leq 0.01$). Unlikely genotype combinations leading to double recombinations over short genetic distance in a few cases were removed by MERLIN (Abecasis et al. 2002).

Linkage Analysis: Nonparametric linkage was examined by MERLIN using Whittemore and Halpern's S_{all} statistics and Kong and Cox's LOD score transformation (Abecasis et al. 2002; Kong and Cox 1997; Whittemore and Halpern 1994). Linkage Disequilibrium (LD) has been shown between tightly linked markers on the Illumina Linkage IV SNP panels (Sawcer et al. 2005) and treating these as independent markers can inflate LOD scores (Huang et al. 2004). MERLIN overcomes this problem by clustering markers that are in LD and estimating the resulting haplotype frequencies to perform multipoint linkage analysis with the composite markers (Abecasis and Wigginton 2005). We implemented the 'rsq 0.05' constraint which requires a pair-wise LD measure of $r^2 < 0.05$ between adjacent markers to create marker clusters; as suggested by a comprehensive simulation study (Levinson and Holmans 2005).

Genetic Heterogeneity Test: Since the IMAGE project is a multi-country

cooperative project, background genetic heterogeneity can be a concern for molecular genetic studies with such mixed samples. Using data from our previous investigation of 51 ADHD candidate genes in the IMAGE sample (Brookes et al. 2006) we found that there was a major allele frequency difference between those collected by the Israeli centers found that there was a major allele frequency difference between those collected by the Israeli centers and the rest of and the rest of the IMAGE sample (Neale et al., submitted). A moderate difference was observed between the Northern European group (Belgium, Germany, Holland, Ireland and UK) and the Southern European group (Israel, Spain and Switzerland). There were only very small and non-significant differences among the Northern European groups; the Israeli group showed the most variation from the Northern European group. A moderate difference was observed between the Northern European group (Belgium, Germany, Holland, Ireland and UK) and the Southern European group (Israel, Spain and Switzerland). There were only very small and non-significant differences among the Northern European groups, while the Israeli group showed the most variation from the Northern European group. Considering that there are only 3 families from each country of Belgium, Spain and Switzerland in this linkage study, we combined them with families from other countries according to the above observations. The Belgium families were merged with the Dutch families (3+57). The Spanish and Swiss families were merged with the Israeli families to form a Southern European group (3+3+18). To account for the major allele frequency difference

found in Israeli families we also tested them as a separate subgroup. Therefore we had 6 subgroups in the genetic heterogeneity test: Holland (57+3), Germany (13), UK (25), Ireland (12), Israel (18) and the Southern European group including the Israeli group (3+3+18).

To examine the potential impact of locus-specific genetic heterogeneity on our observed linkage signals, we repeated the nonparametric linkage analysis in reduced samples by dropping each of the 6 test subgroups in turn. The departures of LOD scores in the reduced sample from those obtained in the full sample may reflect the excluded subgroup's contribution to the linkage signal: a significant LOD score increase in the reduced sample suggests the excluded subgroup makes little or no contribution to linkage at the locus, while a significant LOD score decrease suggests that the excluded subgroup makes a significant contribution to linkage at the locus. The chance of observing such a LOD score departure was evaluated by examining LOD scores from 10,000 repeated nonparametric linkage tests in random samples with the same number of families. To account for the inclusion of 6 dropped groups in the test of heterogeneity, a further simulation considered the LOD score distribution generated from 6 dropped groups for each of 10,000 simulated datasets.

RESULTS

Figure 1 illustrates the nonparametric linkage multipoint LOD scores on the autosomes and the X-chromosome. Table 2 lists the genomic regions showing at least nominal linkage signals (multipoint LOD>1.0) and the markers associated with the peak scores. There are two suggestive linkage signals with LOD scores exceeding

2.0 on chromosome 9 ($Z=2.76$; $LOD=2.13$; $p=0.0009$) and chromosome 16 ($Z=3.61$; $LOD=3.1$; $p=0.00008$). Detailed linkage signal profiles at these two regions are shown in Figure 2. Nominal linkage signals

were found on chromosome 2, 11, 14, 21 and the X chromosome. Table 3 lists the overlap with other linkage studies on ADHD.

Table 2: chromosome regions with $LOD>1.0$

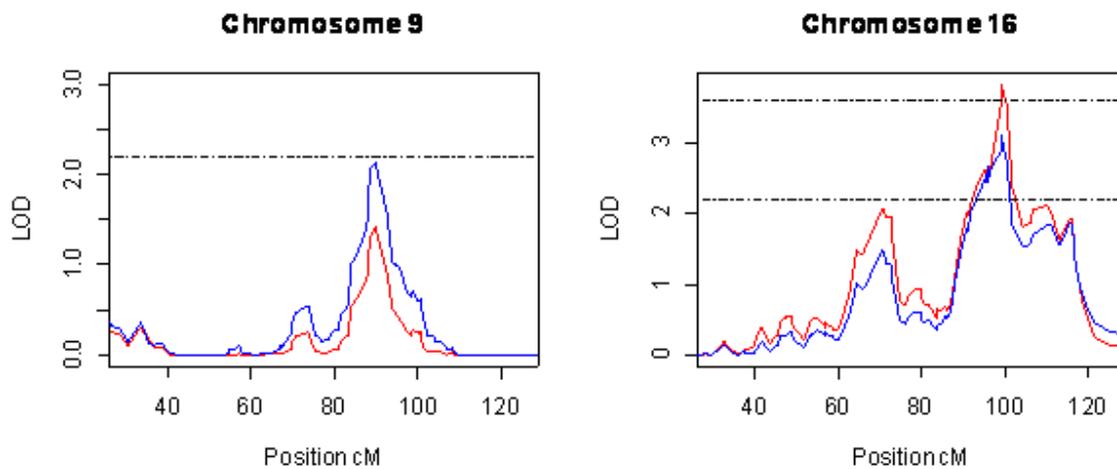
Chromosome	Marker	Physical position	Genetic distance	LOD	
				Full	Non-Is
2	rs1510834	13694789	34.5	1.64	1.37
2	rs2033866	174914536	181.5	1.16	0.85
9	rs7043803	88533215	90	2.13	1.43
11	rs630759	62933285	69	1.31	0.89
14	rs1361525	94339381	100	1.75	1.57
16	rs424074	77792088	99	3.1	3.82
21	rs875060	42817300	61.4	1.53	0.99
X	rs728186	137867027	141.9	1.25	0.94

Table 3: Comparison of the main findings emerged in this study with previous studies.

Chromosome	IMAGE		UCLA		Netherlands		Germany	
	Location	LOD	Location	MLS	Location	MLS	Location	LOD
2	34.5	1.64						
2	181.5	1.16	171	1.00 ^a				
9	90	2.13	93	1.45	132.9	2.05	99.7	0.68
11	69	1.31	72.1	1.17 ^b				
14	100	1.75						
16	70.7	1.51						
16	99	3.1	≈95cM	>1.0	≈100cM	>1.0		
21	61.4	1.53						
X	141.9	1.25						

a. Single point LOD score

Table 4 lists the results of the tests of genetic heterogeneity. It was noted there was a significant difference in DSM-IV symptoms counts for ADHD between subgroups, with the German group significantly higher with an average of 17.21 ($p=0.002$) and the Dutch group significantly lower on 16.14 ($p=0.01$) compared to remaining samples. There were no differences in age or gender between the groups. In our genetic heterogeneity test on chromosome 9, dropping the German group leads to a significant LOD score increase ($p=0.025$). At chromosome 16, there was significant LOD score increase ($p=0.035$) or decrease ($p=0.012$) when the Israeli group or the English group was dropped respectively. These observations were not significant when adjusted for the test of 6 groups in the tests of heterogeneity.

Figure 2 Nonparametric multipoint LOD scores on chromosome 9 and 16.**Figure 2** nonparametric multipoint LOD scores on chromosome 9 and 16 in full sample and northern European subgroup. The blue lines are based on the full sample. The red lines are based on the northern European subgroup. The dash lines are threshold values for suggestive (LOD=2.2) and significant linkage (LOD=3.6) suggested by Lander & Kruglyak (1995)**Table 4:** Genetic heterogeneity test on chromosome 9 and 16

Sample ^a	Families ^b	DSM-IV ADHD score		Chromosome 9			Chromosome 16		
		average	P ^d	LOD	P ^e	P ^e _adjusted	LOD	P ^f	P ^f _adjusted
Full	134	16.39	--	2.13	--	--	3.1	--	--
SE	24	16.24	0.27	1.27	0.155	--	3.43	0.098	--
IL	18	16.35	0.58	1.43	0.156	--	3.82	0.035	0.17
EN	25	16.69	0.09	2.27	0.18	--	1.42	0.012	0.23
DE	13	17.21	0.002	2.82	0.025	0.18	2.87	0.433	--
IR	12	16.29	0.91	2	0.318	--	2.75	0.37	--
NL	60	16.14	0.01	1.07	0.332	--	2.9	0.14	--

- SE: Southern European group; IL: Israel; DE: Germany; EN: England; IR: Ireland; NL: the Netherlands;
- A 4df test of equal proportion of females in each group found no significant discrepancy ($p = 0.39$) (data not shown).
- A 4df Kruskal-Wallis rank sum test of equal age in different group found no significant difference ($p = 0.37$) (data not shown).
- A 4df Kruskal-Wallis rank sum test of equal number of ADHD symptoms in the different groups found at least one group was significantly different ($p = 0.0035$). The p-value column is 1df Wilcox test of equal 'ADHD total score' between each subgroup and all the others.
- The frequency of observing a LOD score greater than the observed LOD score in the entire sample at chromosome 9, from 10,000 simulated datasets: P^e=adjusted for single heterogeneity test, P^e-adjusted=adjusted for six heterogeneity tests
- The frequency of observing a LOD score greater than the observed LOD score for the entire sample at chromosome 16, from 10,000 simulated datasets: P^f=adjusted for single heterogeneity test, P^f-adjusted=adjusted for six heterogeneity tests.

DISCUSSION

In this study we performed a whole genome linkage scan with 142 combined subtype ADHD sib pairs and found suggestive linkage on chromosome 9 and 16 with nonparametric multipoint peak LOD scores of 2.13 and 3.1, respectively. No linkage was observed at the most established ADHD linked genomic region of 5p13.

Our highest linkage signal was found on chromosome 16q23. The multipoint nonparametric LOD peaks at 99 cM on the deCODE genetic map in the vicinity of SNP rs424074. This appears to be an entirely different peak from that reported by the UCLA study on chromosome 16p13 at position 34.5 cM on the deCODE map with a LOD score of 3.73 (Ogdie et al. 2003). However both the UCLA sample and the narrow phenotype analysis of the Dutch sample (Bakker et al. 2003) gave nominal linkage signals on 16q ($1.0 < \text{MLS} < 1.5$, no details reported) with positions around 95 and 100 cM respectively. These fall within the $\text{LOD} \pm 1$ confidence interval from the current study. Furthermore, when the raw data from the UCLA and Dutch samples were pooled in a combined analysis, a slightly higher MLS was obtained ($1.5 < \text{MLS} < 2.0$) (Ogdie et al. 2006).

Alongside our main peak on chromosome 16q12 there is another weaker linkage peak of 1.51 at 70.7cM. Although the linkage signal in this region is only suggestive it is worth noting that the noradrenergic neurotransmitter transporter gene (*SLC6A2/NET1*) is located at position 75.5cM. *SLC6A2* has been reported to show association with ADHD in several studies and warrants further

investigation (Bobb et al. 2005; Brookes et al. 2006; Xu et al. 2005).

We found the second highest multipoint nonparametric LOD peak on chromosome 9q22 at position 90cM. Both the UCLA study and the German study found some evidence of linkage at almost the same location with LOD scores of 1.45 and 0.68 respectively. Although the Dutch study also found suggestive linkage ($\text{MLS}=2.05$) at the adjacent 9q33 region the peak position was at 132.9 cM with no overlap of confidence intervals between the two linkage signals.

We were unable to identify any other significant overlaps of linkage peaks with the four affected sibling pair studies and none with the study of Columbian multiplex families. The MGH scan found no significant evidence for linkage across the genome in an affected sibling pair analysis on 217 families and a quantitative trait locus analysis using the number of ADHD symptoms as the phenotype on 260 families (Faraone et al., submitted). Overall, the general lack of consistency across the ADHD linkage scans indicates that there are unlikely to be genes of moderate to large effects shared across the various populations studied. This general finding of inconsistent reproducibility of linkage findings across datasets has been found in many other psychiatric and common complex disorders and could result from a number of factors (Kunzi et al. 2006). It is to be expected that the genetic structure of behavioural disorders such as ADHD will be complex, with a combination of multiple small additive and interactional effects. It is therefore encouraging that the two loci with the highest evidence in our study overlap with

linkage signals from two other studies. This indicates the possibility that one or more genes located in these two regions makes a significant contribution to the risk for ADHD. Whether these regions do indeed contain ADHD risk alleles will only be determined following fine-grade association mapping to identify either common variants of small effect acting across the various datasets, moderate to large effects from less common alleles, or multiple small effects from multiple rare alleles. Assuming that the two main peaks on chromosome 16 and 9 are true linked loci, we estimate that each contributes a genotypic sibling risk (λ_s) of 1.65 and 1.89 respectively, to the overall familial risk of ADHD ($\lambda_s=1/4z_0$, where z_0 is the estimation of sharing 0 allele at the locus (Risch 1990)).

Table 1 lists the major clinical and demographic characteristics of the affected sibling pair samples in the published ADHD linkage studies. Factors that might increase heterogeneity between the studies include differences in the populations sampled, although the German and Dutch samples are from closely related Northern European populations and the UCLA and MGH samples largely consist of families of European ancestry (~80%). The clinical sample studied here consisting of 100% combined type cases is more closely comparable to the Dutch (85%) and the German (69%) than the UCLA (48%) samples, yet both of the top two loci overlap with data from UCLA. It is feasible that our more homogeneous sample of combined type probands might have led to the relatively high LOD scores for these two loci. However the polygenic nature of ADHD genetics and the stochastic

fluctuations originated from small to modest samples can be expected to lead to the pattern of observations accrued so far.

Although all the families in our sample are of white Caucasian descent there is still the possibility that there are frequency differences for functional risk alleles between the IMAGE sampling sites. From the simulations in our study we did find some evidence for significantly different contributions to the linkage signals between countries. Our prior hypothesis was based on the previous study from Neale and colleagues (submitted) using data from 863 gene-based SNPs that showed that the Israeli group had the highest level of discordance of SNP allele frequencies compared to the rest of the IMAGE sample. We therefore initially dropped the Israeli sample from the analysis and found that the linkage signal on chromosome 16 was significantly higher than in the total. We therefore initially dropped the Israeli sample from the analysis and found that the linkage signal on chromosome 16 was significantly higher than in the total sample (3.82 versus 3.1, $p=0.035$).

We then went on to check more systematically for the effect of dropping each country group in turn and found additional significant changes (Table 4). Without the English group the chromosome 16 finding dropped to 1.42 and without the German samples the chromosome 9 findings increased to 2.82. Our initial simulations showed that the chance of observing such peak LOD score departures is low suggesting that significant heterogeneity might exist (Table 4). However since we performed

several such tests we completed a further simulation of 10,000 datasets in which we dropped samples representing the proportions of each of the six country groups, to generate an empirical distribution of 60,000 (6 x 10,000) simulated LOD scores. For the chromosome 16 locus we found that the upper 5% of the LOD scores were ≥ 4.21 and the lower 5% were ≤ 0.96 and for chromosome 9 the upper 5% were ≥ 3.19 and the lower 5% were ≤ 0.53 . The overall levels of significance adjusted for each of the heterogeneity tests were no longer significant, indicating no overall significant evidence of heterogeneity.

Whether the population mixture in this sample has compromised the power of our analysis cannot be clearly delineated until specific alleles that account for the linkage signals have been identified. The overall power of the sample represents the average genetic effect across the entire sample and will therefore be highest for genes that confer risk across all of the sub-populations studied. Less common genetic risk factors that are restricted to one or a few of the sub-populations might still be identified if they confer relatively large risks. It is worth noting that our full sample size is small and the numbers of families from individual countries are even smaller so that random sampling bias may account for the level of observed genetic heterogeneity.

By using the most up to date SNP linkage panel, we were able to extract more than 95% of the information content for linkage throughout the genome. Testing for linkage in larger datasets or a combined analysis with the previous

linkage data usually follows suggestive evidence of linkage. Thus a meta-analysis of all the available linkage data is now warranted. However, if the true susceptibility loci only confer a small increased risk the required sample size to provide sufficient statistical power to consistently find them may be difficult to achieve. In the next phase of this study linkage disequilibrium or association mapping will be used to search for associated alleles and could identify associated alleles that are very close to functionally significant variants. Future work will focus on fine-mapping information across the identified linkage regions as well as scanning more widely for association signals across the whole genome. An important obstacle towards the interpretation of whole genome association results is the multiple test correction. Applying the prior knowledge of genomic linkage may help to prioritize specific regions with lower multiple test correction penalties. Linkage approaches can also be useful for gene finding if the 'common disease multiple rare allele' scenario, where multiple causal alleles exist within genes, holds true.

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