ELECTRONIC LETTER

Independent replication and initial fine mapping of 3p21–24 in Asperger syndrome

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Background: Asperger syndrome is characterised by abnormalities in social interaction as well as repetitive and stereotyped behaviours and interests. The trait is thought to display complex inheritance, but in a subset of families the inheritance resembles the autosomal dominant model. Linkage to 3p14–24 has recently been reported in Asperger syndrome in Finnish families with a maximum multipoint NPL_{all} of 3.32 at D3S2432.

Methods: We have replicated linkage findings to 3p21–24 in 12 new extended Asperger syndrome families. Linkage analyses were performed separately for the 12 new families, and linkage and association analyses were also performed jointly with data from the original genome-wide screen.

Results: Best two point and multipoint logarithm of the odds (LOD) scores in analyses of both data sets were obtained at D3S2432 ($NPL_{all}=3.83$) with both subsets of families contributing to linkage. Association analysis of the combined data set produced a trend towards association with D3S2432 and D3S1619.

Conclusions: This study further validates 3q21–24 as a candidate region for Asperger syndrome.

Asperger syndrome (AS) belongs to the autism spectrum disorders (ASDs) and lies at the high functioning end of the spectrum.^{1–4} It resembles autism as regards rigid patterns of interests, dependence on routines and rituals, and impairment in social interaction. Key features differentiating AS from autism include the later age of onset, the lack of intellectual deficiency, and the presence of generally normal early language development. Additional symptoms, including sleeping difficulties,⁵ motor clumsiness,⁶ prosopagnosia (face recognition difficulties), and unusual sensory responses,⁷ have been observed in clinical studies but are not used as official diagnostic criteria. Studies on the prevalence of AS have yielded results ranging from 0.3 to 48.4 per 10 000.⁸ The substantial variation in prevalence estimates has been explained by small sample sizes and methodological differences.

The aetiology of AS is unknown. In the original description of the syndrome and in subsequent family studies, behavioural features resembling AS have been observed in close relatives suggesting familial aggregation of the trait.⁹ However, no twin studies have been reported in AS. In some families AS is present without other ASDs,^{10–11} whereas in other families AS and autism co-occur pointing to common aetiological factors.^{12–13} In ASDs in general, family and twin studies suggest a strong genetic component with a multifactorial mode of inheritance.¹⁴ Several genome-wide screens have been performed for ASDs resulting in the identification of numerous genetic loci, but only a few regions, including 2q, 7q, 16p, and 17q, have been replicated in independent studies.¹⁵

We have recently reported the first genome-wide screen of AS in Finnish families.¹⁰ The families display a mild ASD

phenotype with autosomal dominant-like inheritance. No cases with autism were present in these families. The best linkage evidence was observed at 3p14–24, where a maximum multipoint NPL_{all} of 3.32 and a two point $Z_{max dom}$ of 2.50 were obtained. Other potential susceptibility regions were identified at 1q21–23 and 13q31–33.

Here we have enlarged the study by analysing an independent set of 12 novel extended AS families with 54 individuals having AS or AS-like symptoms. A total of 58 markers covering nine genomic regions from the second stage of the original genome-wide screen were analysed. Both datasets supported the linkage to 3p21–24; additional fine mapping was also performed in this region.

METHODS

Families

For this study, we examined 12 new Finnish families with up to 10 affected individuals per family (see supplementary fig 1, available at <http://www.jmedgenet.com/supplemental>). Families were recruited via the Hospital for Children and Adolescents, Department of Child Neurology, Helsinki University Central Hospital and the Helsinki Asperger Center, Medical Center Dextra, Helsinki, Finland. The diagnostic procedure was as described earlier,¹⁰ and consisted of a structured interview based on ICD-10 diagnostic criteria,³ the Asperger Syndrome Screening Questionnaire (ASSQ),¹⁶ and the criteria proposed by Gillberg and Gillberg.¹⁷ In addition, questions regarding hypersensitivity to external stimuli, face blindness (prosopagnosia), motor clumsiness and sleeping, and eating disorders, were included in the interview because these traits have been observed to be common in AS.⁷ Most individuals (72%) included in the molecular genetic studies were examined by one of the authors (LvW, TN-vW, SS, TK) and all affected individuals were seen either by one of the authors or by a neurologist at a Finnish Central University Hospital (n = 3). The interviews were videotaped and the diagnosis was finalised only after consensus had been reached by the clinicians (LvW, TN-vW). Family members of the affected individuals who were not seen by any of the authors were assigned unknown status in the statistical analysis.

Autism was present in a pair of male monozygotic twins, one of whom was included in the genotyping and assigned as unknown in the statistical analysis. No other ASDs were present in the families. A previous diagnosis of schizophrenia (MIM 181500) was present in two individuals from two different families. In one individual the diagnosis of schizophrenia was present in addition to AS. The other subject had no overlapping features of AS and was not included in the analyses.

Abbreviations: AS, Asperger syndrome; ASD, autism spectrum disorder; LD, linkage disequilibrium; LOD, logarithm of the odds; PCR, polymerase chain reaction; PPL, posterior probability of linkage

Two liability classes were used in the statistical analyses. Individuals fulfilling ICD-10 criteria for AS were classified in diagnostic category 1 (LC1), while individuals with AS like features who did not fulfil all ICD-10 criteria were included in a broader diagnostic category 2 (LC2). A total of 42 individuals were included in LC1. Twelve additional individuals with milder symptoms were included in LC2, resulting in a total of 54 affected individuals in this diagnostic category.

Statistical analyses were performed using the newly collected material presented here and then combined with data for the families used in the original study.¹⁰ The combined family material consists of 29 families including 114 affected individuals in LC1 and 136 affected individuals in LC2. The family material is summarised in table 1. These studies have been approved by the ethical committees of the Hospital for Children and Adolescents of Helsinki University Central Hospital and the National Public Health Institute, Helsinki, Finland. Informed written consent was obtained from all participating individuals or their parents.

Laboratory procedures

The 54 microsatellites from the loci that resulted in $Z_{\max} > 1.5$ in the initial stage of the original study¹⁰ located at 1q21–23, 3p14–24, 3q25–27, 4p14, 4q32, 6p25, 6q16, 13q31–33, and 18p11 were analysed using data from the 12 new families. Four new markers were included at 1q23.3–25.1 to extend the coverage of this region towards the telomere. The new markers were selected from the Marshfield Medical Research Foundation map (<http://research.marshfieldclinic.org/genetics/>) and the UCSC Genome Browser (<http://genome.ucsc.edu/>, July 2003 assembly) and genotyped in all families. Primer sequences and physical map locations of the markers were obtained from the UCSC Genome Browser. The average marker spacing was 3.5 cM. The region on 3p21–24, showing linkage evidence in both the original and new datasets, was further analysed by adding 13 new microsatellite markers located between D3S1283 and D3S3660. The markers were selected from the UCSC Genome Browser and, together with the markers from the replication stage, resulted in an average marker spacing of 1.5 cM on 3p21–24.

DNA was extracted from frozen blood samples with the Puregene DNA purification system (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. Polymerase chain reaction (PCR) for microsatellite analysis was performed as described elsewhere.¹⁰ PCR products were pooled and electrophoresed on an ABI 3730 Automatic DNA sequencer (Applied Biosystems, Foster City, CA). GeneScan-500 LIZ Size Standard (Applied Biosystems) was used as an internal size standard in every sample. Fragments were genotyped with GeneMapper 3.0 software (Applied Biosystems). Genotypes were validated by two individuals independently. Genotype errors were checked using PedCheck 1.1¹⁸ prior to statistical analysis. In the

analyses of the new families, controls with known genotypes from the original genome-wide screen were used to ensure compatibility between the genotypes in both datasets.

Statistical analyses

The two point maximum logarithm of the odds (LOD) scores under homogeneity were calculated using the MLINK program of the LINKAGE package.^{19–20} The HOMOG program was used for tests of heterogeneity and for the calculation of the proportion of linked families (α).²¹ The ANALYZE program was used to conduct these analyses.²² Allele frequencies were estimated using DOWNFREQ 2.1. Analyses were performed using both dominant and recessive models with high penetrance and a low phenocopy rate.¹⁰ Disease allele frequencies were 0.003 for the dominant model and 0.054 for the recessive model. An affected only approach was used in all analyses, that is, all individuals were assigned either affected or unknown status.

Both parametric and non-parametric multipoint calculations were performed using GENEHUNTER v.2.1_r5 beta.²³ The marker spacing was obtained from the Marshfield foundation map and marker ordering was verified using the July 2003 version of the UCSC Genome Browser. When two markers had the same location on the genetic map, the spacing was derived using the equivalence of 1 Mb to 1 cM.

Association analyses of the fine mapped region on 3p21–24 were conducted using the PSEUDOMARKER 0.9.7 beta program.²⁴ PSEUDOMARKER is suitable for analysing various pedigree structures and is also able to separate the linkage evidence from evidence for linkage disequilibrium (LD). The test statistic for LD given linkage was used, and both dominant and recessive analyses were performed for both liability classes.

RESULTS

Linkage analysis of the new families

The highest two point LOD score in the replication study was obtained at D3S1768 resulting in $Z_{\max} = 1.77$ (LC1, recessive model); $Z_{\max} = 1.21$ was obtained using the dominant model (LC1) at the same marker. The maximum multipoint NPL score was obtained at the same marker resulting in NPL_{all} of 2.80 ($p = 0.0055$, LC2). This is only 3.5 cM proximal to the most interesting location obtained in the original genome-wide screen, where a multipoint NPL_{all} of 3.32 was obtained at D3S2432.¹⁰ Modest linkage evidence ($Z_{\max \text{ rec}} = 1.42$, LC2, recessive model) was obtained at D3S3547 located 6 cM distal to D3S1768. Of other tested markers, only one additional marker resulted in a maximum LOD score > 1.0 ; D4S3001 on 4p15 resulted in $Z_{\max \text{ rec}} = 1.65$ (LC1, recessive model) and a maximum multipoint NPL_{all} of 1.28 ($p = 0.0868$, LC1) was obtained at the same marker (table 2).

Analyses of the combined family material

When the current and the original datasets were analysed jointly ($n_{\text{families}} = 29$, $n_{\text{LC1}} = 114$, $n_{\text{LC2}} = 136$), the most interesting results were obtained at 3p21–24. At this locus both study samples contributed to linkage. The best two point LOD score, $Z_{\max \text{ dom}}$ of 2.94 (LC1, dominant model), was obtained at D3S2432. Altogether, five out of six markers genotyped in this region resulted in $Z_{\max} > 1$ (table 3). A maximum multipoint NPL_{all} of 3.83 was obtained at D3S2432 ($p = 0.0007$, LC1) in the combined dataset (fig 1). The highest parametric multipoint heterogeneity LOD (HLOD) of 3.71 was obtained at 59.36 cM between markers D3S2432 and D3S1768 (LC1, dominant model). The 1-NPL-drop support interval spanned 16 cM.

On 1q21–23, positive LOD scores were observed in a small subset of the new families, but total LOD scores in the new family material remained marginal. D1S484 resulted in $Z_{\max \text{ dom}} = 0.41$ (LC1, dominant model), and flanking marker D1S2705 resulted in the highest LOD score in the new dataset

Table 1 Summary of family material

	New families	Original families	Total
Total number of families	12	17	29
Individuals genotyped	91	119	210
Affected LC1	42	72	114
Male	20	49	69
Female	22	23	45
Affected LC2	54	82	136
Male	26	54	80
Female	28	28	56

New families refers to the 12 newly recruited families and original families refers to the families analysed in the original genome-wide screen.¹⁰

Figure 1 Multipoint NPL_{all} scores on 3p14–24 following individual and joint analyses of the different family data sets. New families refers to the 12 newly recruited families and original families refers to the families from the genome-wide screen.¹⁰ LC1, liability class 1; LC2, liability class 2.

($Z_{max\ dom} = 0.53$, LC1, dominant model). However, the highest overall two point LOD score in the combined analysis ($Z_{max\ dom} = 3.53$, LC1, dominant model) was obtained at D1S484 on 1q23 (table 3). Additionally, two flanking markers distal to D1S484 resulted in LOD scores >2 . The maximum multipoint NPL_{all} of 1.21 ($p = 0.075$, LC1) was obtained at 165 cM, close to D1S1653 located 5.5 cM proximal to D1S484 in the pooled dataset.

Suggestive linkage evidence in the combined dataset was also observed on chromosomes 4, 6, and 13 (table 3). At 4q32, the best two point LOD score was obtained at D4S2368 ($Z_{max\ dom} = 2.06$, LC1, dominant model). Three flanking markers proximal to D4S2368 all resulted in $Z_{max} > 1$. At 6q16, D6S1671 and D6S1021 located 4.3 cM apart resulted in $Z_{max\ dom} = 1.05$ (LC1, dominant model) and $Z_{max\ dom} = 1.60$ (LC1, dominant model), respectively. At 13q31–33, D13S793 resulted in $Z_{max\ dom} = 1.43$ (LC1, dominant model). The maximum multipoint NPL_{all} of 2.07 ($p = 0.011$, LC1) was observed at D13S1271 located 3.23 cM proximal to D13S793. However, the contribution of the new families to the linkage of these loci was small.

The most interesting region on 3p21–24 was fine mapped by adding 13 new markers increasing the total number of markers to 19 in this region. Six of the 13 fine mapping markers resulted in maximum two point $Z_{max} > 1$ (table 3). Two flanking markers, D3S2432 and D3S1619, showed a trend towards association in dominant PSEUDOMARKER analysis resulting in $p = 0.028$ and $p = 0.041$, respectively. The region flanked by D3S2432 and D3S1619 extends for 3 cM and contains 13 known genes according to the UCSC Genome Browser (May 2004 build).

DISCUSSION

Several genome-wide screens have been performed in families with ASDs using various diagnostic criteria and statistical approaches. Although a strong genetic component for autism has been established in twin and family studies,^{14–25} only a few loci have been replicated in independent genome-wide screens.¹⁵ Different approaches have been applied in attempts to improve the likelihood of finding true susceptibility loci. These strategies have involved inclusion of data from subgroups with, for example, obsessive compulsive behaviours²⁶ and delayed speech development, into the analyses.²⁷ Stratification by clinical phenotype has resulted in increased evidence for linkage to 1q for a subset of individuals with obsessive compulsive behaviours and to 2q for a subset of individuals with delayed phrase speech. In the present study, genetic heterogeneity has been limited both by selecting families with AS only and by selecting the families from the isolated Finnish population. Phenotypic assessment of the families in this study and in the original genome-wide screen has been performed by just a few clinicians using uniform diagnostic procedures across the studies. The limited number of families included in this study is compensated by the large number of affected individuals in the families resulting in substantial power in the linkage analysis. The average number of family members included in this study is 7.2 and the average number of affected individuals per family is 4.7. The emphasis in family selection has been on large multiplex families appropriate for linkage analysis and therefore the male to female ratio and incidence of AS in these families might not be representative of a population sample of AS families in general.

Table 2 Markers resulting in $Z_{max} > 1$ in analyses of the new families						
Marker	cM	Z_{max}	Model and LC	θ	α	NPL_{all}
D3S3547	55.11	1.42	Recessive, LC2	0.00	0.79	1.33 ($p = 0.074$, LC2)
D3S1768	61.52	1.77	Recessive, LC1	0.00	0.66	2.80 ($p = 0.006$, LC2)
D4S3001	49.47	1.65	Recessive, LC1	0.00	0.67	1.28 ($p = 0.087$, LC1)

α , fraction of linked families; θ , recombination fraction; cM, centiMorgan; LC, liability class; NPL_{all} , non-parametric multipoint linkage statistics; Z_{max} , maximum LOD score.

The results provide independent replication of linkage to 3p21–24 in AS in new family material. In light of frequent failures to replicate initial linkage findings in complex diseases, we find it encouraging that added linkage evidence for 3p21–24 was observed in our limited replication sample. We believe our results could be attributed to an appropriate study design aiming at limited heterogeneity, both in sample ascertainment and in analyses. When statistical analyses were performed including the families from the original genome-wide screen,¹⁰ a maximum multipoint NPL_{all} of 3.83 was obtained at D3S2432 in the combined material. Both patient datasets contributed approximately equally to this result. In the original study, the most promising evidence for linkage was obtained at D3S2432, whereas the best evidence for linkage using the new families was obtained at D3S1768, only 3.5 cM proximal to D3S2432. This region is gene rich, especially the proximal end of the linkage peak. According to the USCS Genome Browser (May 2004 build), the 1-NPL-drop support interval contains around 100 known genes. The region was further fine mapped in the combined patient material using additional microsatellite markers and analysed for association using PSEUDOMARKER. Two flanking markers, D3S2432 and D3S1619, resulted in suggestive evidence for association. Therefore, we consider the region between D3S2432 and D3S1619 to be a primary candidate region for future fine scale mapping studies in AS.

In two independent genome-wide screens, suggestive linkage to autism on 3p has been obtained from 11 to 22 cM distal to the best AS region.^{13, 28} In combined analyses of the Finnish autism families and publicly available Autism Genetic Resource Exchange (AGRE) families from the heterogeneous US population, the best linkage signal was obtained at 3p24–26; the best marker was located 29 cM distal to the best marker in this study.²⁹ These findings could

result from the signal of the same susceptibility gene or from multiple different genetic factors. Substantial variation in the location of the linkage signal has been shown in simulations of genome scans for complex trait loci even when the linkage is the result of a single genetic signal.³⁰

Except for the region identified on 3p21–24, no other genotyped region was supported by the linkage evidence from both family datasets. However, in the pooled dataset the highest overall two point LOD score in the combined analyses was obtained on chromosome 1q21–23 (D1S484, $Z_{\max \text{ dom}} = 3.53$). The same marker provided the best result in the original genome-wide screen ($Z_{\max \text{ dom}} = 3.58$), and although the new families contributed to linkage only marginally, linkage of AS to this locus cannot be excluded given the limited number of new families. We have previously reported linkage to 1q21–23 in Finnish autism families with a maximum multipoint NPL_{all} of 2.63 at D1S1653 located 5.6 cM from D1S484.¹³ Risch and colleagues³¹ have obtained a maximum multipoint LOD score of 2.15 at D1S1675, located 20 cM from D1S484. Linkage to 1q23–24 in autism has recently been reported by Bartlett and colleagues.³² They re-analysed the data of Yonan *et al*²³ using the posterior probability of linkage (PPL) method and obtained a 55% PPL on chromosome 1 at 183 cM, 13 cM from our most interesting finding on this chromosome. Significant linkage evidence in the region has also been reported in schizophrenia.³⁴ Recently, an association between schizophrenia and eight markers within the gene for carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (CAPON) have been reported.³⁵ Also, an association between the RGS4 gene and schizophrenia has been reported in this region.³⁶

In summary, we have investigated nine initially interesting chromosomal regions in 12 extended families with AS, based on a previously reported genome-wide screen. We obtained linkage evidence on 3p21–24 overlapping with the linkage

Table 3 Markers resulting in $Z_{\max} > 1$ in analyses of the combined family data

Marker	cM	Het	Two point linkage results				Association results, p value
			$Z_{\max}$	Model and LC	θ	α	
D1S2715	159.32	0.72	1.60	Dominant, LC1	0.20	0.95	NA
D1S2721	161.05	0.74	2.08	Dominant, LC1	0.20	1.00	NA
D1S506	163.34	0.46	1.04	Dominant, LC1	0.26	1.00	NA
D1S2125	163.34	0.48	1.10	Dominant, LC2	0.22	1.00	NA
D1S1653	164.09	0.70	1.46	Dominant, LC1	0.22	1.00	NA
D1S484	169.68	0.74	3.53	Dominant, LC1	0.14	1.00	NA
D1S2705	170.84	0.78	2.24	Dominant, LC1	0.22	1.00	NA
APOA2	170.84	0.74	2.09	Dominant, LC1	0.20	1.00	NA
D1S1677	175.62	0.73	1.02	Dominant, LC1	0.30	1.00	NA
D1S431	182.35	0.80	1.05	Dominant, LC1	0.26	1.00	NA
D3S3547	55.11	0.72	1.18	Recessive, LC2	0	0.52	0.818
D3S2432	57.92	0.82	2.94	Dominant, LC1	0	0.61	0.028
D3S1619	60.98	0.80	1.04	Recessive, LC1	0.18	0.98	0.042
D3S1768	61.52	0.78	1.23	Recessive, LC1	0.18	1.00	0.094
D3S1611	61.52	0.67	1.04	Dominant, LC1	0	0.38	0.127
D3S1298	62.05	0.87	1.68	Dominant, LC1	0.06	0.54	0.349
D3S3685	67.94	0.85	1.99	Dominant, LC1	0.14	0.79	0.966
D3S3678	68.47	0.76	1.37	Recessive, LC1	0.18	1.00	0.107
D3S1767	69.9	0.65	1.54	Recessive, LC1	0.08	1.00	0.390
D3S2456	70.61	0.69	1.05	Dominant, LC1	0.14	0.64	0.520
D3S1766	78.64	0.73	1.91	Dominant, LC1	0.18	1.00	NA
D4S3001	49.47	0.79	1.00	Recessive, LC2	0.16	1.00	NA
D4S3033	161.04	0.72	1.85	Recessive, LC2	0.12	1.00	NA
D4S1598	161.91	0.73	1.23	Recessive, LC1	0.18	1.00	NA
D4S3046	162.47	0.74	1.01	Dominant, LC1	0.22	1.00	NA
D4S2368	167.55	0.74	2.06	Dominant, LC1	0.18	1.00	NA
D6S1671	107.88	0.90	1.05	Dominant, LC1	0.30	1.00	NA
D6S1021	112.20	0.71	1.60	Dominant, LC1	0.26	1.00	NA
D13S793	76.26	0.73	1.43	Dominant, LC1	0	0.39	NA
D13S1271	79.49	0.70	1.06	Dominant, LC1	0.28	1.00	NA

Markers in bold are from the fine mapping of 3p21–24. Heterozygosity was determined from the data using DOWNFREQ.

α , fraction of linked families; θ , recombination fraction; cM, centiMorgan; Het, heterozygosity; LC, liability class; NPL_{all}, non-parametric multipoint linkage statistics; $Z_{\max}$, maximum LOD score.

signals in the original study. These results are further supported by evidence of association in the region. Therefore, we consider 3p21–24 to be a primary candidate locus for future fine scale mapping of AS.

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ELECTRONIC-DATABASE INFORMATION

Supplementary fig 1 is available at <http://www.jmedgenet.com/supplemental>. The Marshfield Medical Research Foundation map is available at <http://research.marshfieldclinic.org/genetics/> and the UCSC Genome Browser can be found at <http://genome.ucsc.edu/>.

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