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Fine mapping of the chromosome 2p12-16 dyslexia susceptibility locus: quantitative association analysis and positional candidate genes *SEMA4F* and *OTX1*

Clyde Francks[a], Simon E. Fisher[a], Richard K. Olson[b], Bruce F. Pennington[c], Shelley D. Smith[d], John C. DeFries[b] and Anthony P. Monaco[a]

[a]*Wellcome Trust Centre for Human Genetics, University of Oxford, UK;* [b]*Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado;* [c]*Department of Psychology, University of Denver, Denver, Colorado;* [d]*Center for Human Molecular Genetics, University of Nebraska Medical Center, Omaha, Nebraska, USA*

Correspondence to A.P. Monaco, Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, UK. Tel: +44 1865 287502; fax: +44 1865 287650; E-mail: anthony.monaco@well.ox.ac.uk

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A locus on chromosome 2p12-16 has been implicated in dyslexia susceptibility by two independent linkage studies, including our own study of 119 nuclear twin-based families, each with at least one reading-disabled child. Nonetheless, no variant of any gene has been reported to show association with dyslexia, and no consistent clinical evidence exists to identify candidate genes with any strong a priori logic. We used 21 microsatellite markers spanning 2p12-16 to refine our 1-LOD unit linkage support interval to 12cM between D2S337 and D2S286. Then, in quantitative association analysis, two microsatellites yielded *P* values < 0.05 across a range of reading-related measures (D2S2378 and D2S2114). The exon/intron borders of two positional candidate genes within the region were characterized, and the exons were screened for polymorphisms. The genes were Semaphorin4F (*SEMA4F*), which encodes a protein involved in axonal growth cone guidance, and *OTX1*, encoding a homeodomain transcription factor involved in forebrain development. Two non-synonymous single nucleotide polymorphisms were found in SEMA4F, each with a heterozygosity of 0.03. One intronic single nucleotide polymorphism between exons 12 and 13 of SEMA4F was tested for quantitative association, but no significant association was found. Only one single nucleotide polymorphism was found in OTX1, which was exonic but silent. Our data therefore suggest that linkage with reading disability at 2p12-16 is not caused by coding variants of SEMA4F or OTX1. Our study outlines the approach necessary for the identification of genetic variants causing dyslexia susceptibility in an epidemiological population of dyslexics. Psychiatr Genet 12:35–41 © 2002 Lippincott Williams & Wilkins

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INTRODUCTION

Dyslexia is a common neurological syndrome that manifests most obviously as a disorder of reading (Smith *et al*., 1996). The syndrome has a strong but complex genetic component (DeFries *et al*., 1987; Smith *et al*., 1996; Olson *et al*., 1999; Fisher *et al*., in press), and evidence for susceptibility loci on chromosomes 1, 2, 3, 6, 15 and 18 has been reported (Fisher & Smith, 2001; Nopola-Hemmi *et al*., 2001; Fisher *et al*., 2002). Two independent genome-wide linkage studies, using very different analytical approaches, have provided convergent evidence that a dyslexia susceptibility locus maps to chromosome 2p12-16 (Fagerheim *et al*., 1999; Fisher *et al*., 2002). Fagerheim *et al*. (1999) analyzed a single four-generation pedigree with a qualitatively defined affection status, and reported a non-parametric linkage ($P = 0.0009$) for a region of 2p15-16, which was the strongest linkage in that genome screen. Fisher *et al*. (2002) analyzed a sample of 119 nuclear sib-pair families drawn from the Colorado twin study of reading disability (DeFries *et al*., 1987; Olson *et al*., 1999), and tested for linkage directly to quantitative reading-related traits. Using one analytical approach based on partitioning of trait variance components, a quantitative trait locus (QTL) on 2p12-16 again provided the strongest evidence for

linkage in the genome ($P = 0.0008$ with a word-recognition measure). The present study was performed using the latter sample of 119 Colorado families. The robust detection of the 2p12-16 QTL by the linkage studies of both Fagerheim *et al.* (1999) and Fisher *et al.* (2002) suggests that the locus is likely to have an important effect in a broad population of dyslexics.

According to Genemap99 (website: http://www.ncbi.nlm.nih.gov/genemap99/), the 2p12-16 region contains 68 known genes, plus approximately 250 uncharacterized messenger RNAs and expressed sequence tags. To refine linkage mapping of the 2p12-16 QTL, we genotyped 21 microsatellite markers spanning the locus with an average intermarker interval of 1.6 cM. We then performed a recently implemented quantitative sib-pair association analysis (Abecasis *et al.*, 2000) with the 21 microsatellites and four correlated measures of reading disability. Association analysis has the potential to provide greater positional resolution than linkage analysis, since the extent of linkage disequilibrium (LD) reflects many more ancestral meioses (Hartl and Clark, 2001). In addition, the quantitative analytical approach is expected to be more powerful than analysis based on qualitative diagnostic schemes, and to better accommodate the phenotypic and genetic complexity of dyslexia (Cardon *et al.*, 1994, 1995; Fisher *et al.*, 1999; Marlow *et al.*, 2001).

We then decided on a candidate gene screening approach within the region of linkage rather than a higher density LD mapping approach (e.g. using a 5–50 kb intermarker interval) due to the prohibitive number of markers needed to span the locus and the inherent multiple testing problem with this latter approach. The aim was to identify potentially functional variants within candidate genes that could be tested directly for association. Since there are no a priori candidate genes for dyslexia, we selected two positional candidates for study on the basis of their reported functions and patterns of expression. These were Semaphorin4F (SEMA4F) and OTX1.

SEMA4F (SEMAM, SEMAW; OMIM 603706) belongs to the type IV family of membrane-bound semaphorins, which are a family of secreted and membrane-bound proteins that regulate axonal and dendritic growth and guidance (Kolodkin *et al.*, 1993; Nakamura and Kalb, 2000). SEMA4F causes the collapse of retinal ganglion cell growth cones *in vitro*, and is expressed in rat embryonic spinal column, retinal ganglion cells and around the optic nerve, and at high levels in the adult rat central nervous system (Encinas *et al.*, 1999). The expression pattern and functional profile of SEMA4F therefore made it a good candidate for the 2p12-16 QTL, especially since subtle defects of the visual system have been reportedly associated with dyslexia (Eden *et al.*, 1996).

OTX1 (OMIM 600036) encodes a homeobox-containing transcription factor, which is involved in forebrain morphogenesis and the differentiation of neural structures, especially of the cortex (Acampora *et al.*, 1999). OTX1 is also postnatally expressed in the pituitary gland and/or the hypothalamus (Acampora *et al.*, 1998). Mice lacking OTX1 show seizures and brain abnormalities (Acampora *et al.*, 1996), and also transient dwarfism and hypogonadism (Acampora *et al.*, 1998), suggesting an involvement in the prepubescent control of some major developmental and reproductive hormones. In this respect, OTX1 might relate to a proposed link between foetal hormone levels and reading disability (Smith *et al.*, 1996). More generally, the involvement of OTX1 in brain patterning and corticogenesis made it a good candidate gene for dyslexia.

MATERIALS AND METHODS

The family sample

One hundred and nineteen nuclear twin-based families were drawn from an ongoing study by the Colorado Learning Disabilities Research Centre (DeFries *et al.*, 1987; Olson *et al.*, 1999). School records from 27 Colorado districts were used to identify twins. Parental permission was then sought to examine the twins' files for evidence of reading problems. If at least one member of a twin pair showed a positive school history of reading problems, both twins and any other siblings were invited to the University of Colorado where they were administered a battery of psychometric tests. The standardized test scores were used directly for quantitative linkage analysis. Details of tests are presented in Olson *et al.* (1999) and Fisher *et al.* (2002). Monozygotic twin pairs were excluded from the present study sample, although if additional sibs were available for a MZ pair, one of the MZ pair and any additional non-twin siblings could be included. The 119 families yielded 180 total pairs. The mean age for the twins and siblings was 11.6 years (range, 8–19 years).

Microsatellite markers

Microsatellites were assigned genetic positions in Haldane centimorgans based on data from Genethon (website: http://www.genethon.fr/), the Cooperative Human Linkage Centre maps (v2hd.abi.sexave, v8c8.recmin.v1.abi.sexave, Recombination-Min;

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website: http://www.lpg.nci.nih.gov/CHLC/), and the Marshfield genetic maps (website: http://www.marshmed.org/genetics/contents.htm). The relative positions of markers (Fig. 1) were verified using published physical mapping data (Whitehead contigs 2.4, 2.5; website: http://www-genome.wi.mit.edu/; Resch *et al*., 1998; Kirschner *et al*., 1999; Stone *et al*., 1999). Genotyping was performed for all siblings and available parents (the majority) in the sample. Semi-automated fluorescent genotyping and data checking and handling were performed using techniques described previously (Fisher *et al*., 1999).

Quantitative linkage analysis

Multipoint Variance Components linkage analysis was performed using GENEHUNTER 2.0. (Pratt *et al*., 2000). Variance Components analysis was performed using a single trait mean, with no QTL or polygenic dominance variance components, and with no manual setting of the mean.

Quantitative association analysis

The sib-pair quantitative association method of Abecasis *et al*. (2000) partitions allelic mean effects into orthogonal between-sibship and within-sibship components, and the within-sibship component forms the basis of the association test. The test is therefore analagous to the transmission disequilibrium test (Spielman *et al*., 1993), insofar as it is family based and robust to population stratification. As well as an allelic mean effect, linkage is simultaneously modelled at the locus using trait variance components, which are conditional on multipoint identity-by-descent (IBD) sharing. For a locus that contributes to trait variance, the linkage variance component is reduced while the allelic association effect is increased (Abecasis *et al*., 2000). Sib-pair quantitative association analysis was performed using the QTDT suite of applications (Abecasis *et al*., 2000). GENEHUNTER 2.0 was used to output multipoint IBD data with which to model the variance components, and the programs PRELUDE and FINALE (from the QTDT package) were used for formatting data. Parental genotype data was used in calculating IBD sharing. One thousand transmission permutations were run in order to obtain unbiased empirical significance levels for the association results, and the multi-allele version of the application (QTDT-MULTI) was used for multi-allelic microsatellite markers.

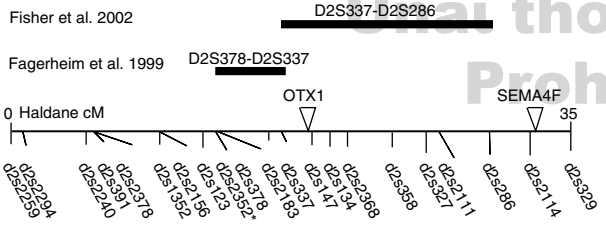

FIGURE 1. Localization of the 2p12-16 locus by dyslexia linkage studies. Localizations correspond to 1-LOD linkage support intervals, and are shown above the markers used in the present study. The positions of OTX1 and Semaphorin4F (SEMA4F) are indicated. Fisher *et al*. (2002) performed genome-wide linkage analysis in the present study sample, and the present study refined the interval to 12 cM. Fagerheim *et al*. (1999) performed a genome-wide screen of a single extended pedigree (see text for details). * Petryshen *et al*. (2000) reported preliminary results from a Canadian family sample that included a single-point LOD of 3.00 using quantitative linkage analysis of marker D2S2352 and a measure of spelling disability.

In silico gene characterization

Starting data in the Genbank database (website: http://www.ncbi.nlm.nih.gov/) on human SEMA4F comprised two sequenced cDNAs (NM_004263.1 and AB021292) obtained from adult hippocampus and forebrain cDNA libraries. Sequence homology searches of the National Center for Biotechnology Information (NCBI) non-redundant database (website: http://www.ncbi.nlm.nih.gov/BLAST) were carried out using both human SEMA4F cDNA clones as probes, and both yielded the same three human genomic sequenced clones with virtually 100% homology; AC006544, AC006543, and AC007387 (Genbank). SEMA4F maps 982 kb proximal to D2S2114 according to the draft sequence contig NT_005428 (Genbank), consistent with our own *in silico* mapping analysis (data not shown). Human SEMA4F has 14 exons (numbered 2–15). Human SEMA4F cDNA clone AB021292 (Genbank) is an alternatively spliced isoform skipping exons 5 and 6, and causing a frameshift, and human SEMA4F cDNA clone NM_004263.1 (Genbank) is an in-frame alternatively spliced isoform skipping exons 5–8 (not exons 5–9 as recorded in the NCBI Genbank entry). A sequence comparison using BLAST between the human SEMA4F cDNA clone AB021292 and the human genomic clone AC006544 enabled the intron–exon structure of human SEMA4F to be constructed for exons 2–4 and 7–15. The rat SEMA4F cDNA clone NM_019272 (Genbank) contained all SEMA4F exons, and sequence homology between this clone and the human genomic clone AC006544 enabled the human sequences of exons 5 and 6 to be determined.

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The resulting *in silico* full-length human cDNA was verified using the program ORF-FINDER (website: http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to match the predicted open reading frame with that previously described (Encinas *et al*., 1999).

A published YAC/BAC contig (Resch *et al*., 1998) of 2p13.3 places OTX1 between the markers D2S147 and D2S2225, and within 2 Mb of D2S147 (Fig. 1). A full-length OTX1 rat cDNA (L32602; Genbank) was used as a BLASTN probe to determine the full-length human cDNA sequence and human intron/exon borders from sequenced human genomic clone AC009501 (Genbank). The resulting sequence was verified against the partial human OTX1 cDNA AB037501 (Genbank).

Exon screening

Each SEMA4F and OTX1 exon, including up to 90 base pairs of flanking sequence, was screened by denaturing high-performance liquid chromatography (DHPLC) in 30 parents from the sibling sample, followed by nucleotide sequencing of variants. DHPLC is a method for distinguishing heterozygous from homozygous polymerase chain reaction (PCR) amplicon samples (Taylor *et al*., 1996). Thirty individuals yield 95% power to detect a single nucleotide polymorphism (SNP) with a rare allelic frequency of 0.05, assuming Hardy–Weinberg equilibrium. Exons of SEMA4F and OTX1 were PCR amplified from parental genomic DNA samples that had been previously shown to yield high-quality genotypes. The hybridized PCR products were screened using the WAVE DNA Fragment Analysis System (Taylor *et al*., 1996), which performs DHPLC with hybridized amplimer duplexes across a range of temperatures. The system can discriminate approximately 90% of SNPs in fragments of between 200 and 500 nucleotides in length. Any samples showing heteroduplex formation were then sequenced to characterize the polymorphisms responsible. Fluorescence-based dideoxy sequencing was performed using BigDye Terminator sequencing kits (Applied Biosystems, Foster City, California, USA) followed by ABI377 polyacrylamide gel electrophoresis (Applied Biosystems) according to standard protocols.

SNP genotyping

The SNP *SEMSNP1* (Fig. 2) was genotyped by restriction fragment length polymorphism analysis in the entire sample. *SEMSNP1* did not cause a known restriction-site change, so a mismatched primer was used for amplification from genomic DNA samples of a 200 base pair region around the SNP, creating an *AccI* restriction site (GTAGAC) for allele2. The sequences were: allele1 genomic sequence 5′-**A**TAGAGCTGGCTCCTGGTTGGC-3′; allele2 genomic sequence, 5′-**G**TAGAGCTGGCTCCTGGTTGGC-3′; mismatched reverse primer, 3′-TCTGGACCGAGGACCAACCG-5′ (where bold represents the SNP and underline represents the mismatch) (the

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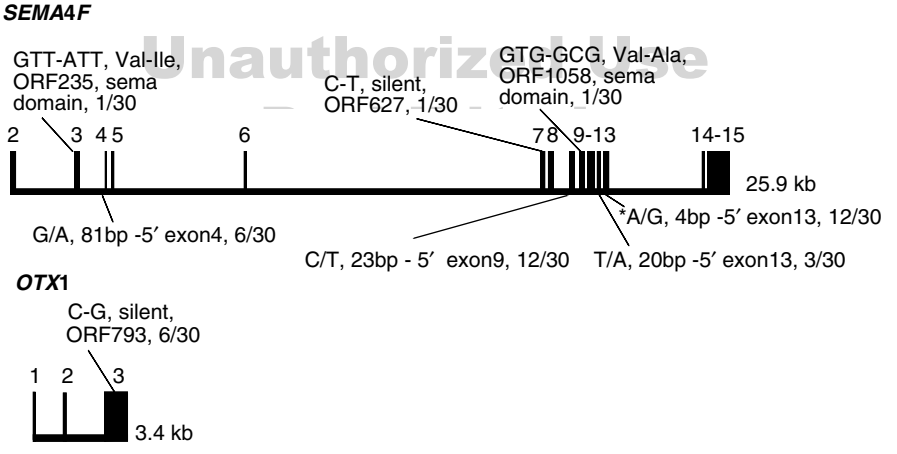

FIGURE 2. Results from intron/exon border characterization, exon screening and single nucleotide polymorphism (SNP) sequencing for Semaphorin4F (SEMA4F) and OTX1. The relative sizes and positions of exons are shown, with exonic SNPs indicated above and intronic SNPs below. Data for each SNP is presented in the order: sequence change (common allele first), amino acid change (if exonic), nucleotide position (in the full-length open reading frame if exonic, or relative to the nearest exon if intronic), protein domain (if exonic and non-synonymous), frequency of heterozygotes in the sample of 30 parents. *This SNP is referred to as *SEMSNP1* in the text, and was genotyped in the entire family sample for association analysis.

forward primer sequence was 5′-TCTTGAAGATCTGGCCTTATTCC-3′). Amplicons were digested with *AccI* (NEBiolabs, Beverly, Masachusettes, USA), and the resulting products were subject to agarose electrophoresis.

RESULTS

Quantitative linkage analysis

Using Variance Components linkage analysis, the 1-LOD support interval was refined from approximately 20 cM following the genome-wide screen (Fisher *et al*., 2002) to 12 cM between markers D2S337 and D2S286 (Figure 1). The peak significance of linkage was not markedly changed from that reported by Fisher *et al*. (2002) (phoneme awareness LOD = 2.3, word recognition LOD = 1.9, orthographic coding LOD = 1.7).

Quantitative association analysis

Results of quantitative sib-pair association analysis are presented in Table 1. Suggestive association was observed for marker D2S2378 (empirical $P = 0.004$ for a word-recognition measure, empirical $P = 0.004$ for a phonological decoding measure), and marker D2S2114 also showed association with three of the measures (empirical $P < 0.05$). Unadjusted P values are shown for this analysis (see Conclusions).

Gene characterization and exon screening

We found that the human SEMA4F gene comprises a 2.3 kb open reading frame distributed over 26.4 kb of genomic sequence, with 14 exons (Figure 2). SEMA4F maps 982 kb proximal to D2S2114 (Figure 1), which was one of the microsatellites to show association. Human OTX1 comprises a 1.065 kb open reading frame with three exons distributed over 3.4 kb of genomic sequence (Figure 2). OTX1 maps between the microsatellite markers D2S337 and D2S147 (Figure 1). Figure 2 summarizes the results from the intron/exon border characterization and exon screening of SEMA4F and OTX1. Only two non-synonymous SNPs were found in SEMA4F, each with a heterozygosity of 0.03, which was too low to perform association analysis. Only one SNP was found in OTX1, which was exonic but silent (6/30 heterozygous).

SNP genotyping and association analysis

To yield enough power to detect significant family-based association, we decided that a SNP should have heterozygosity > 0.2 (assessed from the 30 parents) to qualify for genotyping in the whole sample, unless the SNP was one of several different polymorphisms predicted to change the protein. The SNP *SEMSNP1* (Figure 2) was an A/G change four-nucleotides -5′ to SEMA4F exon 13, for which 12/30 individuals were heterozygous (40%). Unlike the sequence around this nucleotide, the four-nucleotides -5′ position is free to vary for eukaryotic exons in general (Stephens and Schneider, 1992), and therefore unlikely to alter RNA splicing. Nonetheless, *SEMSNP1* was genotyped in the entire sample to test for phenotypic association, since the SNP might have been in linkage disequilibrium with an undetected functional but non-coding polymorphism regulating SEMA4F expression or

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TABLE 1. Quantitative sib-pair association analysis of microsatellite markers

Marker	cM[a]	Reading-related measure[b]			
		WR	PA	OC	PD
D2S2259	0	0.088			
D2S2240	2.4			0.003	
D2S2378	2.9	0.004			0.004
D2S2352	14.1		0.061		
D2S378	15.4				0.035
D2S337	18.6			0.039	
D2S2111	34.5		0.095		
D2S286	36.9			0.049	
D2S2114	38.8	0.078	0.015	0.03	0.043

Empirical unadjusted P values < 0.1 derived from quantitative sib-pair association analysis of 21 microsatellites. Nine markers yielded $P < 0.1$. WR, word recognition; PA, phoneme awareness; OC, orthographic choice, PD; phoneme decoding.
[a]Distance from D2S2259 is given in Haldane cM.
[b]These measures of reading-related abilities have all shown heritabilites > 0.5 in the Colorado twin study (Olson *et al.*, 1999). The inter-trait correlations were high (range, 0.49–0.84), but twin data has shown that these traits can have independent as well as shared genetic variance (Olson *et al.*, 1999). It is therefore logical to test each measure for association.

alternative splicing. After genotyping *SEMSNP1* in the entire sample, the allelic frequencies were determined to be 0.18 : 0.82, with no deviation from Hardy–Weinberg equilibrium ($\chi^2 = 0.036$), yielding heterozygosity = 30%. No significant association was found using quantitative sib-pair analysis of *SEMSNP1* with measures of reading disability (all pointwise empirical $P > 0.1$). Note that an intronic SNP -5′ of exon9 (Figure 2) had a heterozygosity of 40% (12/30) and therefore also met criteria for genotyping. However, the same 12 individuals were heterozygous for this latter SNP as for *SEMSNP1*. The two SNPs were therefore assumed to be in tight linkage disequilibrium, with the rare alleles of both located on the same chromosomes, and hence equivalent for association analysis.

CONCLUSIONS

Twenty-one microsatellite markers spanning the 2p12-16 dyslexia QTL were used to refine the region of linkage in our sibling sample from approximately 20 cM to 12 cM between markers D2S337 and D2S286. The positional concordance between our result and that of Fagerheim *et al*. (1999) (see Figure 1) is encouraging for an etiologically complex trait like dyslexia.

The microsatellites were then tested for quantitative association with four correlated measures of reading disability, and association $P < 0.05$ with at least two measures were found for the markers D2S2378 and D2S2114. Bonferroni correction would not be appropriate for analysis using linked markers and correlated phenotypic measures, and might obscure any real signal in samples of this size. However, an inevitable consequence of not making an adjustment is that false-positive associations at $\alpha = 0.05$ are likely to be observed. For the 2p12-16 locus, this can be partly compensated by ignoring P values < 0.05, which occur for only one phenotypic measure (Table 1), since all of these measures show linkage to some degree at this locus. D2S2378 and D2S2114 are more than 25 cM apart and are not in linkage disequlibrium with one another in this sample (data not shown), and therefore the association results for one marker must represent type I error, assuming that the 2p12-16 linkage is due to only one locus. These association results await confirmation in independent samples.

We decided to move directly to a positional candidate gene screening strategy rather than attempting to saturate the locus with more markers for linkage disequilibrium mapping. Large-scale efforts are currently in progress to identify minimal panels of SNPs that identify common ancestral haplotype blocks separated by recombination hotspots (Johnson *et al*., 2001). Until such information becomes available, the completion of a pure association-based approach remains impractical for most loci. We determined the genomic structures of candidate genes SEMA4F and OTX1, and the exons of both genes were screened for polymorphisms. However, no coding variants of SEMA4F or OTX1 were identified that could account for the linkage with reading disability observed at 2p12-16. The possibilities remain that non-coding regulatory variants or major rearrangements at these loci could cause the 2p12-16 linkage, although our strategy will be to continue screening exons of other candidate genes in the region before investigating these possibilities.

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