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A candidate region for Asperger syndrome defined by two 17p breakpoints

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Asperger syndrome (AS) is a mild form of autistic disorder characterised by impairment in social interaction as well as a restricted pattern of behaviour, interests, and activities. Two patients with AS and balanced translocations t(13;17) and t(17;19), respectively, were identified. Fluorescent *in situ* hybridisation (FISH) analysis with chromosome 17 specific clones to metaphase chromosomes from both patients showed that the chromosome 17 breakpoints are located within a 300 kb region at 17p13. The region spans 14 known genes. The expression of these genes was analysed in lymphoblastoid RNA derived from the patients and healthy control individuals. The CHRNE, DKFZP566H073, LOC90048, PFN1, SPAG7, KIAA0909, ZNF232 and KIF1C genes showed similar levels of expression in cell lines with the translocations when compared with cell lines with normal karyotype. No expression was detected for the MINK, GP1BA, SLC25A11, ENO3, FLJ10060 and USP6 genes in any of the cell lines. The close physical relation of the two 17p breakpoints suggest a common genetic aetiology for the phenotype in the patients. Structural and functional analysis of the genes located around the two 17p breakpoints in t(13;17) and t(17;19) patients may reveal candidate sequences for the AS phenotype.

European Journal of Human Genetics (2002) 11, 189–195. doi:10.1038/sj.ejhg.5200939

Keywords: Asperger syndrome; autism; balanced translocation; candidate region

Introduction

Autistic disorder includes a broad spectrum of manifestations and symptoms. Childhood autism (Kanner syndrome) is characterised by abnormalities in development of social interaction, communication and imagination, which are apparent before the age of 3 years. Mental retardation, aggression and absence of speech are often present in severe cases. Other neurological disorders may overlap with childhood autism (autism spectrum disorders) with different degree of manifestations (see Gillberg and Billstedt¹ for a

review). Asperger syndrome (AS) has been conceptualised as a mild form of autistic disorder (high-functioning autism). The essential features of Asperger syndrome are severe and sustained impairments in social interaction and the development of restricted repetitive patterns of behaviour, interests and activities. Asperger syndrome is diagnosed almost exclusively in individuals with normal or high level of intelligence. A genetic factor behind autism has been proposed, and search for autism candidate regions has been performed by different groups.^{2,3} Several genomic screen studies have revealed susceptibility loci for autism on chromosomes 1, 2, 5, 6, 7, 8, 13, 16, 18, 19 and X.^{4–11} Cytogenetic abnormalities involving chromosomes 7 and 15 in patients with autism have been reported³ but molecular analyses of these chromosomal regions have failed to reveal alleles or genes associated with autism.^{12–15}

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Received 22 February 2002; revised 25 November 2002; accepted 27 November 2002

In this report, we present the clinical, cytogenetic and molecular findings in two non-related patients with AS. The two patients carry apparently balanced reciprocal translocations involving 17p. We suggest that the phenotype in these patients results from a direct or a positional effect of the breakpoints on chromosome 17.

Materials and methods

Case reports

Case 1 The first patient was a 19 year old man, born after 42 gestational weeks by elective caesarean section. Birth weight, length, and head-circumference were 3560 g, 51 cm, and 36.5 cm, respectively. He was the first child to a 23-year-old mother. He had two younger half-sisters, one of whom has autism. The other half-sister has ADHD (Attention Deficit-Hyperactivity Disorder) and DCD (Developmental Coordination Disorder). The mother has a history of anorexia nervosa and obsessive personality traits but autism was excluded. At the age of 11 years, the patient met Gillberg criteria for AS¹⁶ according to ASDI-interview¹⁷: (i) social impairment; (ii) narrow interests; (iii) repetitive routines; (iv) speech and language peculiarities; (v) non-verbal communication problems; and (vi) motor clumsiness (scores 3,3,2,3,2,1; cut-off scores 2,1,1,3,1,1). At the age of 13 years, a new evaluation showed that he met 11 of 16 criteria for autistic disorder according to DSM-III-R (cut-off score 8) and, at age 19 years, he also met the ADI-R algorithm criteria for childhood autism (scores of 12, 11 and 7 for social, communication and behaviour problems, respectively).¹⁸ Cytogenetic analysis revealed that he has an apparently balanced translocation t(13;17)(q14;p13). Both parents and the half-sisters have normal karyotypes.

Case 2 The cytogenetic findings of the second patient was described previously¹⁹ and we performed re-evaluation of the patient's clinical conditions. The patient is a 20-year-old man. He was the third-born child to a 34-year-old mother and had three healthy brothers. The parents are healthy and without a family history of mental or behavioural disorders. The pregnancy and delivery were uneventful. Birth weight, length, and head-circumference

were 3280 g, 50 cm, and 34 cm respectively. During the first year, the patient had fever-related seizures. Speech- and motor development during infancy was normal. At the age of 20 years, he met criteria for AS¹⁶ in all subdomains according to ASDI-interview: Social impairment, narrow interests, repetitive routines, speech and language peculiarities, non-verbal communication problems and motor clumsiness (scores 3,1,1,3,3,1; cut-off scores 3,1,1,3,1,1). He met five DSM-III-R criteria for autistic disorder (cut-off score 8). He did not fulfil the ADI-R algorithm criteria for childhood autism (scores of 7, 4 and 3 for social, communication and behaviour problems, respectively). Cytogenetic analysis revealed an apparently balanced translocation t(17;19)(p13.3;cen). Both parents have normal karyotypes.

Genomic probes

The chromosome 17 specific YAC clones were obtained from the Fondation Jean Dausset–C.E.P.H. YAC/BAC services. The PAC and BAC clones were obtained from Resource Centre/Primary Database (RZPD) of the German Human Genome Project. Single-copy genomic probes for Southern hybridisation were obtained by PCR amplification using BAC DNA. The list of primers used for the amplification of probes is presented in Table 1. The PCR fragments were separated in a 1% agarose gel and purified by QIAquick gel extraction kit (Qiagen).

Cytogenetic and FISH studies

Standard chromosome preparations were made either from PHA-stimulated peripheral blood lymphocytes or from EBV-transformed B-lymphocytes.

Fluorescent *in situ* hybridisation was performed essentially as previously described.^{20,21} Purified PAC and BAC DNA were labelled with digoxigenin-16-dUTP (Boehringer Mannheim) by nick-translation. Total yeast DNA containing a specific YAC was purified by the spheroplast method²² followed by digestion with *EcoRI* and labelling with digoxigenin-16-dUTP by nick-translation. The probes were detected by the application of a single layer of rhodamine labelled anti-digoxigenin (Boehringer Mannheim).

Table 1 Primers used for amplification of PCR-derived probes applied for mapping of the 17p translocation breakpoint in t(17;19) patient

Probe name	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)
ENO1	TCAAGTCATCCCCACAATC	CTTCGAACCCAACATCCT	771
PFN1	TTGAGGGTGCTTTTTTGTGTG	GGTTTTGGCAGCAATAAGGG	783
SLC25A11	TTCCTCCCCTCCCTGTGTC	AGTACCATCAGCCCCAGTCA	617
GP1BA	GGCTTTGGTGGGGAACCTTG	CAGACCTGCCGAAAGACACA	826
GOOD	CATGCCCTCTTCCCAGATT	GCCAGAGATTGAGAAGGAAC	713
CYBER1	ACGAGGAATGAACATGGCAG	AGTCACGGGTGCTTTATCTT	265
CYBER2	GGCTGGGTTGCTAGAGGG	TCCGCTCTCTGCTCCTCTC	322
CHRNE	AGGCAGAGGGGAGAGAGGT	ATCGAAGGGGAAGTAGGTG	874
MINK1	CTGGGGGATTCTGGGCTG	CAAGAATGAACAAGCAGCAGA	717

Chromosomes were counterstained using DAPI (Serva), and mounted in Vectashield anti-fading solution (Vector Labs). The slides were analysed with a Zeiss Axioscope microscope. Images were merged using a cooled CCD camera (Photometrics) and the IPLab software (Vysis).

Southern blot hybridisation

Genomic DNAs were isolated from peripheral blood obtained from patients and normal control individuals using standard procedures. Southern blot and hybridisation were carried out essentially as described.²³ Ten μg of DNA was digested with the appropriate restriction enzyme; fragments were separated on 0.8% agarose gel, transferred to Hybond-N+ membrane (Amersham), and hybridised with probes labelled with ^{32}P -dCTP by random priming.

Northern slot hybridisation

Total RNA was purified from EBV-transformed B-lymphocytes by TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. The patients and normal control RNAs were transferred to Hybond N+ membrane (Amersham) using Bio-Dot SF microfiltration unit (Bio-Rad Laboratories) by the method described in Sambrook *et al.*²³ The RNAs were applied to the membrane in separate slots (20, 10, 5, 2.5, 1.25 μg , 625, 312 and 156 ng, respectively). The membranes were hybridised to oligonucleotide probes derived from the mRNA sequences (Table 2).

The oligonucleotides were labelled with ^{32}P -ATP by the following procedure: The reaction mixture containing 5 μl of an oligonucleotide (10 ng/ μl), 5 μl 10 \times T4 polynucleotide kinase buffer (New England BioLabs), 2 μl T4 polynucleotide kinase (10 U/ μl), 5 μl γ - ^{32}P -ATP (10 mCi/ml) and 33 μl H₂O was incubated at 37°C for 75 min followed

by a purification from unincorporated nucleotides with QIAquick nucleotide removal kit (Qiagen). Oligonucleotides with a specific activity of 7–15 $\times 10^8$ cpm/ μg were used for hybridisation.

Pre-hybridisation was carried out in a solution containing 20% formamide, 5 \times Denhardt's reagent, 10 mM phosphate buffer pH 7.0, 0.1% SDS and 100 ng/ μl sonicated salmon sperm DNA at 37°C for 30 min. The labelled oligo-probe (50 ng) was then added to the hybridisation solution and incubated at 37°C overnight. In case of 28S oligo (Table 2), 500 ng of the cold oligonucleotide was also added.²⁴ The post-hybridisation washing of the membranes was performed twice in 2 \times SSC/0.1% SDS at 42°C for 15 min and once in 0.1 \times SSC/0.1% SDS at 42°C for 5 min. The signals were analysed with a PhosphorImager.

The amount of RNA was quantified by hybridisation with oligonucleotide probes to 28S RNA and β -actin (ACTB) RNA. In order to identify possible DNA contamination in the RNA sample, membranes were hybridised to oligonucleotides complementary to the oligo-probes.

Results

Mapping the chromosome 17 translocation breakpoints

The cytogenetic analysis of the two Asperger syndrome patients revealed apparently balanced translocations t(13;17)(q14;p13) and t(17;19)(p13;cen), respectively. Both translocations were found to be *de novo*, since the parents had normal karyotypes. Further mapping of the chromosome 17 breakpoints was performed by FISH analysis. Five YAC clones on 17p13 were initially chosen as probes (Whitehead Institute/MIT Center for Genome Research; http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map). Fluorescent *in situ* hybridisation of the YAC clones to metaphase chromosomes from the patients showed that both 17p breakpoints were located in the region between clones 898_a_10 and 961_c_4.

Further FISH analysis with PAC and BAC clones located in the region restricted both breakpoints to a region flanked by the clones RP11-177H5 and RPCIP-1193O3. The region is covered by the chromosome 17 working draft sequence segment NT_010823. Using the GenBank (<http://www.ncbi.nlm.nih.gov>) information, we constructed a physical map (Figure 1) spanning the 17p breakpoints. The analysis revealed that the 17p breakpoint derived from the t(17;19) was located in the overlapping region of the clones RP11-81A22, RP11-177H5 and RP11-305G1 (Figure 2), whereas the clones RP11-46I8, CTD-2013F15 and RP11-333E1 spanned the 17p breakpoint derived from the t(13;17) (Figure 1). The FISH analyses gave no indications of other rearrangements or deletions.

Southern blot analysis was performed in order to map the 17p breakpoint position in the t(17;19) patient more precisely. Genomic probes derived from the NT_010823 sequence were hybridised to DNAs from the patient and normal controls digested by *Apa*LI, *Eco*RI or *Sph*I restriction

Table 2 Oligonucleotide probes used for Northern slot hybridisation

Gene name	Oligonucleotide sequence (5'-3')
MINK1	TCTTTTCAGCAACACAAAGTCTGCGGGC
MINK2	TCCTCACCAATTGCTCGCTTATAGCTT
CHRNE	CAGAAGCAGATGTTGTCAAGGGCATTTC
GP1BA	CTGAAATTGTGTGCTGTGTGTGTAAG
SLC25A11	TGAGTCCAGTAAGAACTGCTTGGATTG
DKFZP566H073	TTCTCACTATTCCACACCATGTTCA
LOC90048	AGAGAGGAACAAGGTGAAGCACCT
PFN1	TGCTCTTGGTACGAAGATTTATGCTAA
ENO3	CTCTTAGTTCCAGAGCCTCATAGATAC
SPAG7	TGTAGTATGCTCCTCTCGATCTTGTTC
KIAA0909	TTTCGATGATCTGCTTGGCTAGTGAGA
KIF1C	TCTTCATTCTCATCTCATGATCCTGG
FLJ10060	TCTTCTGGAACTGAAGACCTTCTAGT
ZNF232	TCAGTAATGGGTGGTGTGGCAATGAT
USP6	GCTTGTGAATACCACTGCGTGTGTGTA
28S	AACGATCAGAGTAGTGGTATTTTACC
ACTB	ACAACAATGTGCAATCAAAGTCCTCG

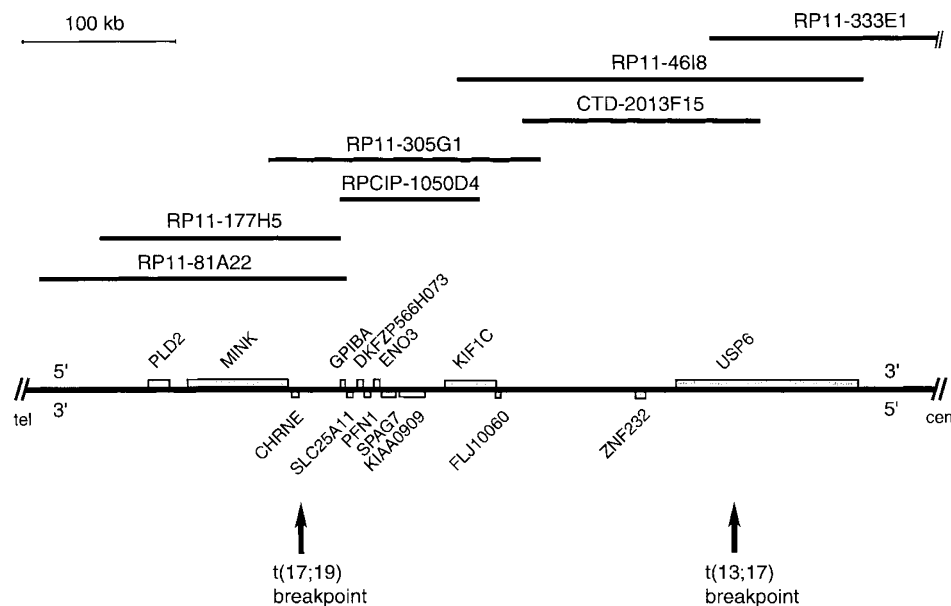


Figure 1 Physical map of the chromosome 17 region, which spans the breakpoints. The relative positions of PAC and BAC clones used for FISH mapping of the breakpoints are shown (top). The positions of the two breakpoints are indicated by arrows (bottom). The left breakpoint is mapped to a 0.7 kb region and the right to a 25–30 kb region. Genes located in the region are indicated by grey boxes. Genes transcribed towards the centromere are indicated above the line whereas genes transcribed in the opposite direction are placed below the line.

Table 3 Genes selected for the investigation of their expression level in t(17;19) and t(13;17) patients

Gene	Protein	Known function	Reference
MINK	Misshapen/NIK-related kinase	A member of the germinal center kinase (GCK) family. The expression is up-regulated during the postnatal development of the mouse brain. The gene has 2 alternative transcripts (MINK1 and MINK2).	25
CHRNE	Cholinergic receptor, nicotinic, epsilon polypeptide	A subunit of acetylcholine receptor at mature mammalian neuromuscular junctions.	26
GP1BA	Platelet glycoprotein Ib, alpha polypeptide	Platelet glycoprotein responsible for platelet adhesion to subendothelium	27
SLC25A11 (SLC20A4)	Solute carrier family 25, member 11	Carrier of 2-oxoglutarate across the inner membranes of mitochondria	28
DKFZP566H073	Hypothetical protein	Unknown function	
LOC90048	Hypothetical protein	Unknown function (probably an alternative splicing product of DKFZP566H073)	
PFN1	Profilin 1	Inhibitor of the actin polymerisation. The function is not well understood. PFN1 is ubiquitously expressed in mouse while its homolog PFN2 is expressed at high level in brain only.	29
ENO3	Enolase 3	Muscle-specific glycolytic enzyme (2-phospho-D-glycerate hydrolyase)	30
SPAG7	Sperm associated antigen 7	Unknown function	31
KIAA0909	Hypothetical protein	Unknown function	
KIF1C	Kinesin family member 1C	A microtubule-based motor protein. KIF1C is involved in vesicle transport from the Golgi apparatus to the endoplasmic reticulum	32
FLJ10060	Hypothetical protein	Unknown function	
ZNF232	Zinc-finger protein 232	Unknown function	33
USP6	Ubiquitin-specific protease 6	Protease expressed in cancer cells of various origin (Tre-2 oncogene)	34

enzymes. Abberant bands were detected in the patient DNA digested with *Apa*LI and *Eco*RI enzymes when hybridised with the CHRNE probe (Table 1). The result indicates that the breakpoint is positioned within a 0.7 kb region located between the CHRNE and GP1BA genes and, 0.8 kb away

from the 5' end of the CHRNE gene. From the Southern analyses there were no indications of complex rearrangements or deletions.

In summary, the results indicate that the two 17p breakpoints in the patients are separated by 250–

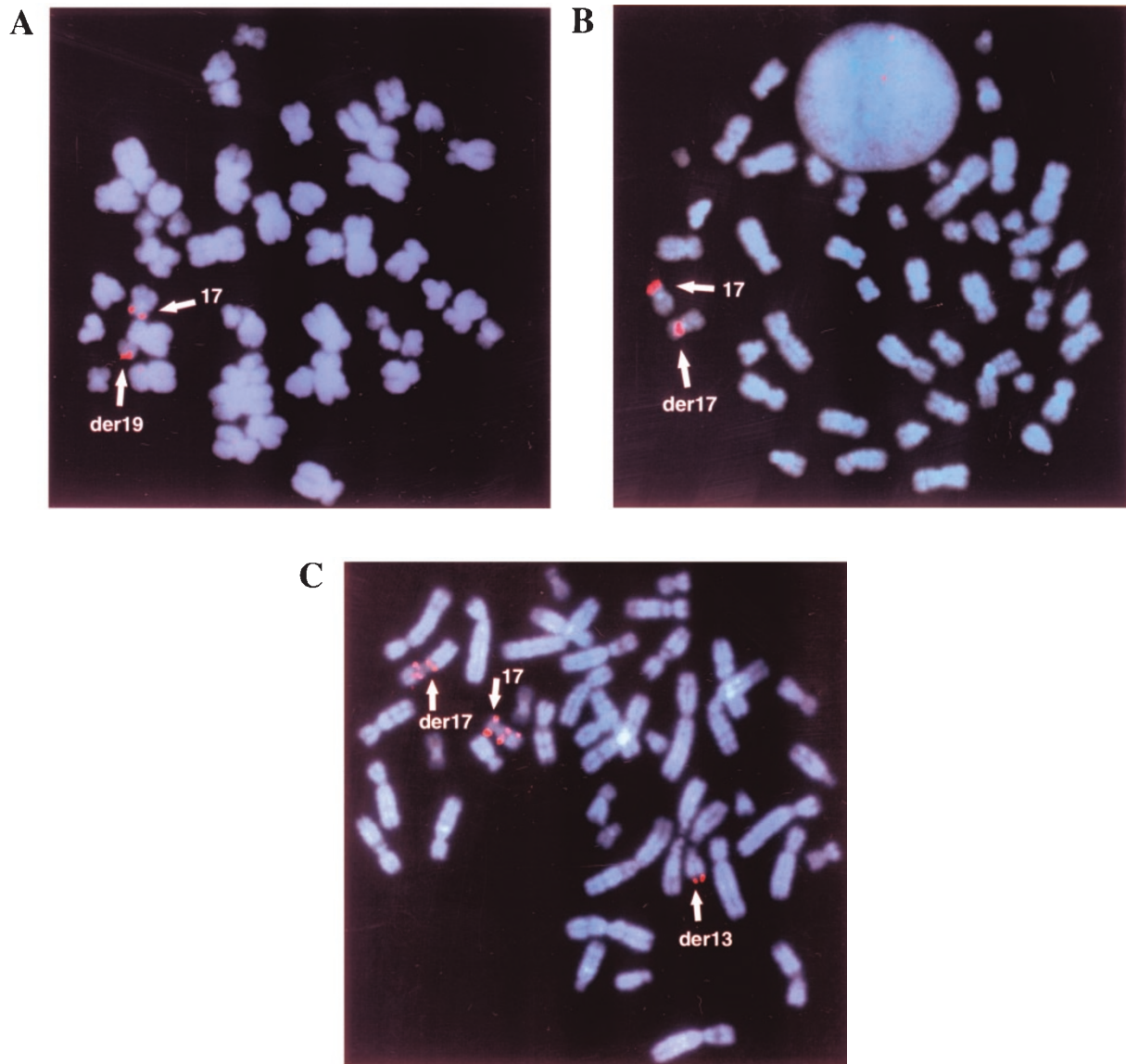


Figure 2 Fluorescent *in situ* hybridisation of the BAC clones (red signals) and to metaphase chromosomes from the patients. (A) Hybridisation of the BAC clone RP11-81A22 to chromosomes from the patient with t(17;19). The clone is detected on the native chromosome 17 and chromosome 19 derivative (arrows). (B) Hybridisation of the BAC clone RP11-305G1 to chromosomes from the patient with t(17;19). The clone is detected on the native chromosome 17 and chromosome 17 derivative (arrows). (C) Hybridisation of the BAC clone RP11-4618 to chromosomes from the patient with t(13;17). The clone is detected on native chromosome 17 as well as the chromosome 13 and 17 derivatives (arrows). Additional red signals on the native 17 and der17 correspond to homologous sequences present on the chromosome 17.

300 kb according to GenBank sequence data (Figure 1). The telomeric 17p breakpoint is located close to the 5' end of CHRNE gene between the CHRNE and GP1BA genes whereas the centromeric 17p breakpoint maps to a 25–30 kb region within the USP6 gene. The breakpoints are located approximately 2 Mb centromeric to the LIS1 gene associated with the Miller-Dieker syndrome.

Expression studies

The region, which spans the chromosome 17 breakpoints in both patients, contains 14 known genes (Figure 1). We hypothesise that the similar phenotype in both patients is related to the same gene(s) in the region. Expression analysis of all known genes located between the 17p breakpoints (Table 3) was performed using lymphoblastoid RNA derived from the two patients and six controls. After Northern slot

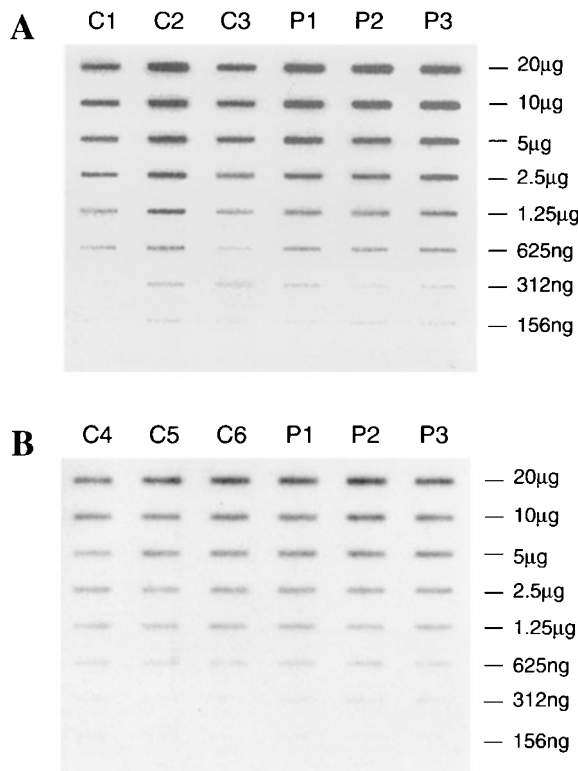


Figure 3 Northern slot hybridisation of the PFN1 oligonucleotide probe (Table 2) to lymphoblastoid RNA from the patients (P) and normal controls (C). (A) Analysis of three independent cell lines from the patient with t(17;19) and three control individuals (C1–C3). (B) Analysis of three independent cell lines from the patient with t(13;17) and three control individuals (C4–C6). The amount of RNA applied per slot is indicated. Similar patterns from Northern slot analysis of RNA from the patients was found for the other genes located between the two chromosomal breakpoints.

hybridisations with oligonucleotide probes derived from mRNA sequences (Table 2), the level of expression was compared between the different cell lines. Total RNA was analysed from three independent cell lines from each patient, and from six control individuals. Expression levels of the CHRNE, DKFZP566H073, LOC90048, PFN1, SPAG7, KIAA0909, ZNF232 and KIF1C genes were found to be similar in the cell lines containing the translocations when compared to cell lines with the normal karyotype (Figure 3). No expression of the MINK1, MINK2, GP1BA, SLC25A11, ENO3, FLJ10060 and USP6 genes was detected in RNA from any of the cell lines.

Discussion

We present here the clinical, cytogenetic and molecular findings in two non-related patients with Asperger syndrome and balanced translocations involving chromosome 17. We found no indications of complex

rearrangements or deletions associated with the two translocations.

An intensive search for genes behind autism has revealed a number of susceptibility loci. However, the results from several genomic screens are inconsistent.^{2,3} Autism is part of a wider spectrum of neuropsychiatric disorders with a considerable phenotypic variation (autism spectrum disorders)¹ and the clinical diagnosis may be difficult. Differences in allele frequencies between various human populations as well as different definitions for the various autism spectrum disorders may be additional factors that influence the results. Autism is also a common associated problem of disorders including behaviour abnormalities such as Tourette syndrome, ADHD and obsessive-compulsive disorder. Therefore, it is possible that the phenotypic variation within autism and autism spectrum disorders reflects a difference in genetic factors, which stand behind these abnormalities.

In this report, we have analysed two patients diagnosed with Asperger syndrome without major associated symptoms. The patients presented here share two important features. Firstly, both patients have been diagnosed with Asperger syndrome by the same psychiatrists. Secondly, both patients have *de novo* chromosomal abnormalities, which involve a relatively small region on chromosome 17. These data suggest that the Asperger syndrome phenotype in both patients may be related to gene(s) or regulatory sequences located in the region. The fact that the mother and a half-sister of one of the patients have a history of behavioural abnormalities, which are not associated with 17p rearrangements, contradicts to this assumption. However, phenotypic differences between these individuals and complex inheritance of such traits raise the possibility that Asperger syndrome in the patient described here is caused by the t(13;17).

Fourteen genes have been mapped to the region between the two 17p breakpoints, and each gene is a potential candidate. In the present study, we investigated the mRNA expression from these candidate genes in cell lines with the translocations and in cell lines with a normal karyotype. Samples of a nervous tissue were impossible to obtain from the patients, and we restricted our analysis to EBV-transformed B-cells. We found no differences in mRNA expression of the genes CHRNE, DKFZP566H073, LOC90048, PFN1, SPAG7, KIAA0909, ZNF232 and KIF1C between cell lines carrying the translocations and normal cells. Expression of the MINK, GP1BA, SLC25A11, ENO3, FLJ10060 and USP6 genes was not detected in any cell line under the conditions used. Further analysis of expression levels in different cell types will be needed for the identification of candidate genes involved in the Asperger syndrome phenotype. The MINK1 and MINK2 genes are known to be involved in mouse brain development, and participate in signal-transduction cascades in nervous system,²⁵ which makes them candidates for further expression analysis.

In summary, we have identified a new susceptibility region for Asperger syndrome on 17p13 based on the molecular characterisation of two chromosome breakpoints. Further studies of the expression and function of genes located in the region may reveal candidate genes or regulatory sequences involved in the disease.

Acknowledgements

We thank Dr Nils-Erik Heldin and Dr Taras Stasyk for advice and technical assistance. This work was supported by grants from the Swedish Medical Research Council, Childrens Cancer Foundation of Sweden, the Swedish Cancer Society, the Söderström-Königska Foundation and the Swedish Medical Society, The Sävstaholm Society, Torsten and Ragnar Söderbergs Fund, The Lundbeck Foundation, the Borgström Foundation, and The Wilhelm and Martina Lundbergs Foundation.

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