The neurobeachin gene is disrupted by a translocation in a patient with idiopathic autism

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Autism is a developmental disorder characterised by a triad of clearly abnormal or impaired development in social interaction and communication, and a markedly restricted repertoire of activity and interests.¹ Its incidence is estimated at about 1/1000 to 1/2000.² Different metabolic and structural brain anomalies have been observed in subjects with autism but these data have not yet led to a single unifying theory on its pathogenesis. In a minority (5–10%) of cases, autism is a symptom of a recognisable disorder such as fragile X syndrome, tuberous sclerosis, or untreated phenylketonuria.³ However, the molecular pathways involved in these disorders have also not contributed to an increased understanding of the pathogenesis of autism. In the majority of cases, the cause of autism is not known but there is strong evidence for a genetic cause. A polygenic inheritance is likely but estimates on the number of interacting genes vary from two to 10.^{4,5} Moreover, it is likely that different combinations of genes are implicated in unrelated subjects.⁶ The identification of genes involved in autism is expected to increase our understanding of the pathogenesis of this disorder. Several large scale linkage studies and follow up analyses have yielded suggestive linkage to several different chromosomal regions. However, neither this approach nor the large number of association studies using candidate genes has resulted in the identification of autism susceptibility genes.⁷ As an alternative approach to identifying candidate genes for autism, we initiated a positional cloning strategy starting with subjects with autism carrying a de novo chromosomal anomaly. In a group of 525 subjects with autism who were karyotyped and had no recognised underlying medical condition, four were found to carry such a de novo chromosomal aberration. In none of them was there a family history of autism. Three had an apparently balanced reciprocal translocation, 46,XY,t(1;15)(p35.3;q24.2), 46,XY,t(5;13)(q12.1;q13.2), and 46,XY,t(14;16)(q12;q24.3), and one subject had a paracentric inversion with karyotype 46,XY,inv(10)(cen;q21.3). The incidence of these aberrations in this population of patients with autism is much higher than would be expected from the low incidence in the normal population of 1/10 000 for a paracentric inversion and 1/2000 for a reciprocal translocation.^{7,8} Together with the de novo origin of the aberrations and negative family histories for autism, this suggests that, at least in some of these subjects, there is a causal relationship between the chromosome aberration and the occurrence of autism. Here, we describe a detailed molecular genetic analysis of a male with autism, with a de novo balanced translocation t(5;13)(q12.1;q13.2).

CASE REPORT

This boy was the only child of healthy, unrelated parents. The family history was negative with regard to autism or other developmental or psychiatric disorders. Pregnancy and delivery were uneventful. Early developmental milestones were

Key points

- A high incidence of de novo chromosomal aberrations in a population of persons with autism suggests a causal relationship between certain chromosomal aberrations and the occurrence of isolated idiopathic autism.
- The neurobeachin encoding gene is disrupted in a patient with a de novo translocation t(5;13)(q12.1;q13.2), idiopathic autism, and no family history of autism.
- The neurobeachin gene is located in a region identified as a candidate region for autism on chromosome 13q.
- Expression data strongly suggest an important role of neurobeachin during brain development.
- Neurobeachin in a candidate gene for autism.

normal; he walked at 15 months and first words appeared at 12 months. Phrasing speech occurred at 18 months. Already during infancy and early childhood, peculiar behaviour like an obsession with music, absence of joined attention, a limited play repertoire, and insistence on sameness were noticed, but this did not lead to further investigation until primary school age. At 3 years, attending nursery school, he did not speak for an entire year and had very little social contact with his peers. Hand flapping was noted and gross motor skills including swimming and cycling were delayed. At primary school, learning difficulties became apparent. These were mainly related to poor abstract reasoning and poor visuospatial abilities. He functioned at a borderline mental level (WISC full scale IQ of 86, with verbal IQ 94, performance IQ 80). Problems in social interactions remained present throughout his school career. At the age of 14, our subject had a child psychiatric assessment. At that time, he had no social contact with his peers, except a cousin. He had marked impairment in social interactions and reciprocity; eye contact was sporadic. Speech was monotonous and with inappropriate volume. He used to talk to himself in public and language content was echolalic and stereotypical. The subject stuck very much to routine, even pestering routines that were difficult to stop, and had stereotypical emotional reactions. The diagnosis of autism was made by means of a Dutch standardised questionnaire for autism spectrum disorders, the AUTI-R,⁹ and confirmed by an experienced child psychiatrist (LS), who found impairments in the three core domains of autism according to the DSM-IV criteria,¹ and made the clinical diagnosis of autism. At the age of 15 years, physical examination was normal. There were no dysmorphic features. Height and head circumference were on the 90–97th centile and weight was above the 97th centile. Neurological examination was normal, as was a brain MRI.

METHODS

Strategy

The position of the chromosomal breakpoints was determined by means of fluorescence in situ hybridisation (FISH) using yeast artificial chromosome (YAC) clones, and further refined by the use of bacterial artificial chromosomes (BAC) and P1 artificial chromosomes (PAC) contained within the breakpoint spanning YAC. A cosmid library was screened using DNA probes from the breakpoint spanning BAC clone. The cosmids obtained were ordered in a contig based on sequence analysis of the cosmid ends and by PCR to confirm overlapping cosmids. A cosmid spanning the breakpoint was then identified by FISH. Sequence comparison of the cosmid ends and the known DNA sequence of the breakpoint spanning BAC allowed DNA probes for Southern blot analysis to be defined, to confirm the location of the breakpoint.

Genomic resources

Selection of the YAC clones was based on the STS based Map of the Human Genome and on YAC and radiation hybrid screening data of the Human Physical Mapping Project at the Whitehead Institute/MIT Genome Center. UCSC Genome Browser¹⁰ was used to select BAC and PAC clones within the breakpoint spanning YAC. BAC and PAC clones were obtained from Invitrogen Life Technologies. Cosmids were provided by the UK Human Genome Mapping Project Resource Centre.

FISH

FISH analyses were performed on metaphase spreads obtained from peripheral white blood cells and from an EBV immortalised cell line from the patient. BAC/PAC/cosmid clones or inter-Alu PCR products of YACs were biotinylated with biotin-11-dUTP (Roche Diagnostics GmbH) by nick translation using the Bio Nick Labeling System (Invitrogen Life Technologies). For the double colour FISH experiments, one probe was labelled with digoxigenin-11-dUTP (Roche Diagnostics GmbH) and the second probe with biotin-11-dUTP.

Southern blot analysis

Genomic DNA of the patient and control was treated with spermidine, digested with the enzymes *Bam*HI, *Eco*RI, *Hind*III, or *Pst*I, and electrophoresed on an 0.8% agarose gel. Southern blotting and hybridisation were carried out according to standard protocols.¹¹ The filter was hybridised with a 385 bp PCR product from intron 2 of the *hNbea* gene, purified with QIAquick gel extraction kit (Qiagen), and radiolabelled with α -³²P-dCTP using the Megaprime DNA labelling system (Amersham Pharmacia Biotech). Primers used to generate this probe were 5'-TCCATTGTTTCATCACCACTTGTGG-3' and 5'-CATGTAACAAGTCAATCTCCTCTTCCCC-3'.

Northern blot analysis

Total cellular RNA was extracted with the RNeasy Midi kit (Qiagen) from EBV cell lines of the patient and controls. Northern blot analysis was performed according to standard procedures.¹¹ To investigate the expression pattern, a human 8-Lane MTN™ Blot (Clontech) was used. A probe was generated by RT-PCR on Total Human Brain RNA (Clontech), purified with QIAquick gel extraction kit (Qiagen), and radiolabelled with α -³²P-dCTP. Primers used to generate this 410 bp probe were 5'-TCGGTGGCATTATGAGCATCAGAAC-3' and 5'-AATGGGATGCAGGTTAGGTTCAAAGC-3'.

In situ hybridisation

A probe was generated by RT-PCR on Total Human Brain RNA (Clontech), purified with QIAquick gel extraction kit (Qiagen), and cloned in the pGEM-TEasy vector (Promega). Primers used to generate this 369 bp probe were 5'-TGAGGAGTACCAGCGACAAGAGGAG-3' and 5'-TCTCCAC-CCACCAATGTCTCTGG-3'. The cDNA containing plasmid was

linearised and used as template for antisense and sense digoxigenin (DIG) labelled riboprobe synthesis (Roche Diagnostics GmbH). After transcription, the template cDNA was digested by RNase free DnaseI (20U/ μ g).

Embryos of 15 days were dissected and immediately embedded in tissue Tek (Agar Scientific) and then frozen in isopentane in liquid nitrogen; 12 μ m thick tissue sections were prepared using a microtome (−20°C, Microm HM 500 OM) and postfixed in 4% paraformaldehyde-PBS.

The sections were incubated three times with 1 $\times$ PBS then a 1% tritonX-100 (five minutes, room temperature) treatment and an acetylation (15 minutes, room temperature). The sections were then prehybridised for one hour at room temperature in the hybridisation mix (50% formamide, 5 $\times$ SSC, herring sperm DNA 0.5 mg/ml). The riboprobes were denatured for five minutes at 85°C and added to the hybridisation mix (1.25 μ g/ml). Hybridisation was carried out at 65°C for 16 hours. The sections were then washed for 30 minutes in 2 $\times$ SSC (room temperature), one hour in 0.1 $\times$ SSC (65°C), one hour in 2 $\times$ SSC (65°C) and equilibrated for five minutes in buffer 1 (maleic acid 100 mmol/l and NaCl 150 mmol/l, pH 7.5). The sections were then blocked for one hour in buffer 1 containing 1% blocking reagent (Roche Diagnostics GmbH).

For the detection, the sections were incubated for one hour at room temperature with alkaline phosphatase coupled anti-digoxigenin antibody (Roche Diagnostics GmbH) diluted 1:5000 in blocking buffer. Excess antibody was removed by three 20 minute washes in buffer 1, and the sections were equilibrated for five minutes in buffer 2 (Tris HCl 100 mmol/l, NaCl 100 mmol/l, MgCl₂ 50 mmol/l, pH 9.5).

Colour development was performed at room temperature (16 hours) in buffer 2 containing NBT and BCIP (Roche Diagnostics GmbH). After staining, the sections were washed in deionised water, dried at room temperature, and mounted in poly-mount (Polysciences Inc).

RESULTS

Molecular analysis

The position of the breakpoints on chromosome 13q13.2 and 5q12.1 was determined by means of FISH on prometaphase chromosomes of the patient. As shown in fig 1A, the breakpoint was flanked by BACs RP11-307013 (NCBI AL138690) and RP11-66B8 (NCBI AL161902). Cosmids 97e9 (fig 1B) and 107c7 were found to span the breakpoint on chromosome 13. Southern blot analysis with a probe from these cosmids showed rearranged fragments in the DNA of the patient using different restriction enzymes (fig 1C). The breakpoint could thus be localised to a 2.8 kb *Hind*III/*Bam*HI restriction fragment. Sequence analysis of the cosmid ends and the probe used in the Southern blot study showed that the 13q13.2 breakpoint is located in the intron between exon 2 and 3 of the human neurobeachin gene (*hNbea* (NCBI NM015678)) (fig 1A). On chromosome 5q, the breakpoint was localised within the centromeric 125 kb part of BAC RP11-586E1 (NCBI AC113420). In silico analysis (Ensembl Genome Server, UCSC Genome Browser, NCBI-Entrez Genome) showed that this region is devoid of known or predicted genes.

The neurobeachin gene consists of 58 exons spanning 730 kb of the human genome, at the boundary of chromosome 13q13.2-13.3. A CpG island (encompassing 1456 bp of *hNbea* and covering exons 1 and 2) was identified (WebGene). A putative promoter was also found within this region using the Promoter Inspector program (Genomatix). The gene codes for a transcript of 10.8 kb. Since the breakpoint falls in the second intron, distal from the putative promoter, and since no alternative transcripts have been identified lacking the first two exons, the translocation in the current patient is predicted to lead to an absence of neurobeachin expression from this allele.

Figure 1 The neurobeachin gene on chromosome 13 is disrupted by a translocation breakpoint in a patient with idiopathic autism. (A) Physical map of the human chromosome 13q13.2-q12.3 region (UCSC Genome Browser, June 2002 version). The position of the BAC ends (AL138690 and AL161902) and cosmids used for FISH analysis are shown. Arrows indicate the position of the translocation breakpoint with regard to the genomic clone (→, breakpoint more distal; ←, breakpoint more proximal). The position of the Southern blot probe used in (C) and the 2.8 kb *HindIII*/*Bam*HI restriction fragment are indicated on the restriction map of the cosmids spanning the breakpoint. The breakpoint is located within the second intron of the neurobeachin gene. (B) FISH analysis on metaphase spread of the patient shows that cosmid 97e9 (green signal) spans the breakpoint on chromosome 13. Controls are cosmid 84c11 (SUBTEL p., green signal) and a centromeric probe of chromosome 13 (red signal). (C) Southern blot analysis using a probe from the spanning cosmids shows rearranged fragments (indicated by arrow) in the patient (P) compared to a control (C) using different restriction enzymes.

Expression study

Additional evidence supporting a role for human neurobeachin in a neuropsychiatric developmental disorder like autism is its abundant expression in the brain of humans (fig 2A) and mice.¹² A low level of the *hNbea* mRNA was also seen in skeletal muscle, kidney, and heart, whereas no detectable expression was seen in the placenta, lung, liver, or the pancreas. During development, a high expression of *Nbea* was observed in the mouse central nervous system by means of in situ hybridisation (fig 2B) and immunohistochemistry (data not shown). During postnatal brain development in mouse, mRNA levels decrease approximately by 50% during the first 25 days.¹² These expression data suggest an important role of

NBEA in brain development, which concurs with a probable developmental origin of autism.¹³

Northern blot analysis was performed using RNA extracted from the EBV transformed lymphoblastoid cell lines from the patient and from controls, but *hNbea* expression could not be detected in those cell lines (data not shown), so that *hNbea* haploinsufficiency could not be shown in this patient.

DISCUSSION

In this patient with isolated autism, the neurobeachin gene was found to be disrupted by a balanced reciprocal translocation. The neurobeachin gene maps at 26.87 cM near marker D13S624. It is of particular interest that this falls within the 19

Figure 2 Predominant neurobeachin gene expression in the central nervous system. (A) Multiple tissue northern blot (Human MTN Blot, Clontech) using a 410 bp probe from exon 58 of the *hNbea* gene. He(art), Br(ain), Pl(acenta), Lu(ng), Li(ver), Sk(eletal muscle), Ki(dney), Pa(ncreas). (B) In situ hybridisation of an E15 mouse embryo using a 369 bp probe from exon 23 of the *mNbea* gene.

cM region that was identified as a candidate region for autism on chromosome 13q.¹⁴ A maximum MMLS/het score of 2.3 was found in this region between markers D13S217 (17.21 cM) and D13S1229 (21.51 cM). Approximately one-third of the families were found to link to this locus under a recessive model for autism. A follow up study, incorporating information on proband and parental language development, reinforced the finding that this locus may harbour an autism susceptibility gene.¹⁵ However, in contrast to a locus more distal on chromosome 13q that is predominantly attributable to a subgroup of families with autism and language delay, the locus at 21.5 cM is not entirely attributable to this subgroup of families. This is of interest since the patient we studied did not have early language delay.

Human neurobeachin was initially identified in an attempt to isolate novel proteins in neuronal synapses, but was subsequently found to be a neurone specific multidomain protein that is associated with tubulovesicular intracellular membranes throughout the neuronal cell bodies and dendrites.¹² NBEA is an A kinase anchoring protein (AKAP) that recruits the cAMP dependent protein kinase A (PKA) to endomembranes near the *trans*-Golgi network. In addition to its PKA RII subunit binding domain, NBEA has a C terminal BEACH-WD40 sequence module characteristic of the members of the BEACH protein family, such as lysosomal trafficking regulator (LYST, the prototype member and mutated in *beige* mouse and human Chediak-Higashi syndrome), beige-like protein (BGL) or FAN (a signal transduction protein in TNF signal pathway). Although the function of the WD40 repeat in BEACH containing proteins is currently unknown, it is likely that this domain is involved in recruiting the protein to the proper localisation in the cell (for example, FAN).¹⁶ Additionally, a recent study has identified the presence of a pleckstrin homology (PH)-like domain in *hNbea*. Structural analysis and biochemical studies showed that the PH and BEACH domains have strong interactions and form a prominent groove, suggesting these domains may be involved in protein-protein interactions.¹⁷ Further unravelling of the cellular pathway in which neurobeachin functions may lead to finding other candidate genes for autism.

In the past, a number of candidate genes for autism have been identified in genetic syndromes featuring additional physical anomalies or global developmental delay. The relevance of these genes to the understanding of the pathogenesis of idiopathic autism is uncertain. The present patient, however, had isolated autism, without associated

mental retardation or physical anomalies. Therefore, the finding of a breakpoint in the neurobeachin gene in this patient with a *de novo* translocation and isolated autism, together with the predominant expression of *Nbea* in brain and during development, and the chromosomal localisation of the *hNbea* gene in a locus previously implicated in autism strongly suggest that the neurobeachin gene is a candidate gene for idiopathic autism in humans.

Final genetic evidence linking the candidate gene to autism will be obtained by the detection of mutations in this gene in other patients with autism, or the association of polymorphisms in this candidate gene with autism.

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Electronic database information. UCSC Genome Browser (Kent WJ 2002), <http://genome.ucsc.edu/> (for physical map human 13q13.2-q12.3 and 5q12.1 region). Ensembl Genome Server (Sanger Institute), <http://www.ensembl.org/> (for in silico analysis AC113420). National Centre for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/> (for AL138690, AL161902, AC113420 and *hNbea* (NM015678)). NCBI-Entrez Genome, <http://www.ncbi.nlm.nih.gov/entrez/> (for in silico analysis AC113420). WebGene (Institute of Advanced Biomedical Technologies (ITBA)), <http://l25.itba.mi.cnr.it/genebin/wwwcpg.pl> (for CpG analysis *hNbea*). PromoterInspector (Genomatix), <http://genomatix.gsf.de/cgi-bin/promoterinspector/promoterinspector.pl> (for promoter analysis of *hNbea*).

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