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Case report

A case of autism with an interstitial deletion on 4q leading to hemizygosity for genes encoding for glutamine and glycine neurotransmitter receptor sub-units (*AMPA 2*, *GLRA3*, *GLRB*) and neuropeptide receptors *NPY1R*, *NPY5R*

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Abstract

Background: Autism is a pervasive developmental disorder characterized by a triad of deficits: qualitative impairments in social interactions, communication deficits, and repetitive and stereotyped patterns of behavior. Although autism is etiologically heterogeneous, family and twin studies have established a definite genetic basis. The inheritance of idiopathic autism is presumed to be complex, with many genes involved; environmental factors are also possibly contributory. The analysis of chromosome abnormalities associated with autism contributes greatly to the identification of autism candidate genes.

Case presentation: We describe a child with autistic disorder and an interstitial deletion on chromosome 4q. This child first presented at 12 months of age with developmental delay and minor dysmorphic features. At 4 years of age a diagnosis of Pervasive Developmental Disorder was made. At 11 years of age he met diagnostic criteria for autism. Cytogenetic studies revealed a chromosome 4q deletion. The karyotype was 46, XY del 4 (q31.3-q33). Here we report the clinical phenotype of the child and the molecular characterization of the deletion using molecular cytogenetic techniques and analysis of polymorphic markers. These studies revealed a 19 megabase deletion spanning 4q32 to 4q34. Analysis of existing polymorphic markers and new markers developed in this study revealed that the deletion arose on a paternally derived chromosome. To date 33 genes of known or inferred function are deleted as a consequence of the deletion. Among these are the *AMPA 2* gene that encodes the glutamate receptor GluR2 sub-unit, *GLRA3* and *GLRB* genes that encode glycine receptor subunits and neuropeptide Y receptor genes *NPY1R* and *NPY5R*.

Conclusions: The deletion in this autistic subject serves to highlight specific autism candidate genes. He is hemizygous for *AMPA 2*, *GLRA3*, *GLRB*, *NPY1R* and *NPY5R*. GluR2 is the major determinant of AMPA receptor structure. Glutamate receptors maintain structural and functional plasticity of synapses. Neuropeptide Y and its receptors *NPY1R* and *NPY5R* play a role in hippocampal learning and memory. Glycine receptors are expressed in very early cortical development. Molecular cytogenetic studies and DNA sequence analysis in other patients with autism will be necessary to confirm that these genes are involved in autism.

Background

Although there is clear evidence from twin studies and from family studies that genetics contributes to the etiology of autism, it is likely that many different genes are involved [1-3]. Identification of autism candidate genes through linkage analysis is hampered by the extreme genetic heterogeneity in autism and by the fact that there are many more sporadic cases of autism than familial cases. In a number of different genetic diseases, structural chromosome changes (deletions, duplications, translocations or inversions) that segregate with the disease phenotype have served to narrow the search for disease determining genes to specific chromosome regions and subsequently to specific candidate genes. Recently there has been substantial progress made in the identification of genes for X-linked mental retardation by molecular genetic investigation of structural chromosome changes, including microdeletions of the X chromosome. These studies led to identification of candidate genes for mental retardation. Subsequently mutation screening of these candidate genes in subjects without chromosome changes led to definitive identification of genes responsible for X-linked mental retardation. These studies in mental retardation constitute a paradigm for identification of genes that play a role in autism. Our molecular genetic analysis of the deletion on chromosome 4q in the patient described here highlights specific autism candidate genes. These genes are of particular interest since they encode receptors for glutamine and glycine neurotransmitter receptors and receptors for neuropeptides Y.

Case presentation

We report a case of autism in which a chromosome 4q microdeletion was detected.

Pregnancy and family history

The patient, AU0052-0201, was born to a 38-year old mother at 42 weeks of gestation. Pregnancy was uneventful. According to the mother, an amniocentesis performed was normal. Delivery was reportedly induced secondary to oligohydramnios. The birth weight was 7 1/2 pounds (50^{th} percentile); birth length was 20" (50^{th} percentile). At birth, metatarsus adductus and calcaneovalgus of the left foot, bilateral 2^{nd}–3^{rd} toe syndactyly and bilateral cryptorchidism were noted. Family history is non-contributory. The patient is the youngest of three children born to a non-consanguineous Caucasian couple. His elder brother and sister are healthy and developmentally normal.

The patient rolled over at 3–4 months, sat alone by 6 months, stood alone at 15–18 months and walked alone at 20 months. His motor milestones delays were attributed to casting on his left foot for correction of metatarsus adductus.

Dysmorphology evaluation

Minor dysmorphic features noted at 12 months of age were a nose with a flat, broad base, broad tip, slightly short bridge and slightly anteverted nares, bilateral 5^{th} finger clinodactyly and short 5^{th} metatarsals bilaterally. The patient's head circumference was at $<5^{th}$ percentile from ages 1 to 12. However, at 15 years his head circumference was noted to be at the 35^{th} percentile. A neurological exam at age 15 revealed grossly intact cranial nerves. There was no evidence of tics, tremors, or seizures.

Psychosocial assessment

At age 11 years 10 months, the patient satisfied diagnostic criteria for autistic disorder by DSM-IV criteria [4] in an evaluation using the Autism Diagnostic Observation Schedule- Generic (ADOS-G) Module 2, [5] and the Autism Diagnostic Interview- Revised (ADI-R) [6]. The patient test scores revealed impairments in social interaction and communication and increased repetitive stereotypic behaviors. The patient received a composite score of 59 on the Stanford-Binet Scales of Intelligence, Fourth Edition [7].

Language assessment

The patient's first word "dada" was uttered at 2 years of age. At 26 months, his mother reported he said few words and uttered 'animal sounds'. The patient communicated through gestures rather than vocalizations. Assessments of language included the Peabody Picture Vocabulary test-III Form A (PPVT-IIIA) [8] and the Preschool Language Scale-Third Edition (PLS-3). The scores from the language testing at 11 years and 10 months revealed that auditory comprehension age was equivalent to 4 years and 6 months, and expressive communication age was equivalent to 3 years and 10 months.

Routine cytogenetic testing

Karyotype analysis on peripheral blood was carried out at the age of 10 years 11 months. Metaphase chromosomes at 550 band resolution were examined. These studies revealed an interstitial deletion on chromosome 4q. The karyotype was reported as 46, XY del 4 (q31.3-q33). Molecular genetic studies were undertaken to define the limits of the deletion, to determine which genes were deleted in consequence of the deletion and to define the parental origin of the deletion.

Molecular cytogenetic analysis

White blood cells and cultured lymphoblastoid cell lines from the patient were used to produce slides with spreads of metaphase chromosomes and interphase nuclei. A series of linearly ordered BAC (bacterial artificial chromosome) clones were identified from the Human Genome Resources database at http://www.ncbi.nlm.nih.gov[9] for the region of interest on chromosome 4q. The BAC

Table 1: New microsatellite repeat polymorphisms identified in the 4q32-4q34 region.

Marker	Primer sequence	Size of most common allele
Pdgfc106	F- CCC AAG CAG AGT TGA GGG TA R- TGA TTA ACA CTG AGA TAT TTG GCA TT	148–150 bp
612J15	F- CAG GGA CCT GTA GTG TTG TGC R- GGA ACT GAA TTA GGA ACC AGA TAT ACA	118–122 bp
GluR22488	F- GAC TGC CTG CCC CTC TCT R- CCC TCT CTC CTC TCT CAC TCA	98 bp
M6-91472	F- CTC CCC CAA CAC ACA TCT CT R- GCT GGT GAA ATC TCT TCC TAT TAC A	151–153 bp

clones span 4 sequenced contigs and cover about 30 Megabases (Mb) of DNA on chromosome 4 from the 4q31.3-4q32 junction to 4q35.2.

The BAC clones were obtained from Research Genetics-Invitrogen as glycerol stocks and processed according to supplier's protocol. DNA from individual BAC clones was labeled using nick translation and SpectrumGreen dUTP (Vysis TM). Labeled BAC DNA was ethanol precipitated along with human Cot1 DNA to block repetitive sequences. The precipitate was resuspended in hybridization buffer (Vysis). Each clone was then used as a probe in fluorescence in situ hybridization (FISH) experiments on the metaphase chromosomes and interphase nuclei from the patient's peripheral blood lymphocytes and cultured cells. Hybridization and post-hybridization washing of slides was carried out according to the manufacturer's protocols (Vysis). SpectrumOrange dUTP labeled 4q subtelomeric probe was used as a control.

Analysis of polymorphic markers

A total of 21 microsatellite repeat markers were used to define the proximal and distal breakpoints of the deletion. 17 markers were identified from the Human Genome Resources website at http://www.ncbi.nlm.nih.gov[9]. Primer sequences were obtained from the Marshfield Clinic database [10]. The heterozygosity of all these markers, except D4S2427, was obtained from the comprehensive genetic map generated from the Centre d'Etude du Polymorphisme Humain (CEPH) database [11]. Only markers with heterozygosity between 0.70 and 0.90 were chosen for the analysis.

Four new polymorphic markers, Pdgfc106, 612J15, GluR22488 and M6-91472 were defined. They were identified by screening DNA sequences available on the Human Genome Resources website for regions of dinucleotide repeats. Sequences flanking the repeats were used to design primers for amplifying the repeat containing segment. Primer sequences were entered into BLAST http://www.ncbi.nlm.nih.gov/BLAST/[12] to determine that the DNA sequence of each primer set was unique based on current sequence information and that each primer set identified a single locus. Primer sequences for these markers are documented in Table 1. Pdgfc106 primer sequence starts at nucleotide 106763 in Bac RP11-154F14. Primer sequence for marker 612J15 starts at nucleotide 31423 in Bac RP11-612J15. Primer sequence for marker GluR22488 starts at nucleotide 22488 in Bac RP11-392C20. Primer sequence for marker M6-91472 starts at nucleotide 91861 in Bac RP11-368D18.

Primers were ordered from Sigma Genosys with the 5' end of the forward primer fluorescein-labeled. PCR products generated from genomic DNA were electrophoresed on an ABI 377 electrophoresis system with laser detection. The gel peaks representing different alleles were compared to labeled size standards to determine the allele sizes.

Study protocol was approved by the Human Subjects Internal Review Board at University of California, Irvine.

Cytogenetic analysis

Molecular cytogenetic analysis with the BAC clones in FISH experiments revealed a 19 Mb deletion in the patient on chromosome 4 spanning 4q32 to 4q34 (Table 2). Each of the BAC clones used gave a unique signal on chromosome 4q.

Microsatellite analysis

Table 3 summarizes the data from the markers that were informative in this family. The deletion arose on the paternally derived chromosome as a *de novo* event. Non-paternity is unlikely as the patient inherited a paternal allele for all informative markers outside the deleted region and also for markers on other chromosomes (data not shown).

Genes in the deleted region

The deleted region was examined using the Human Genome Resources website at NCBI [9]. The 19 Mb deletion spans five sequenced contigs with 33 genes for which

Table 2: AU0052-0201 FISH data summary

Clone ID	Starts at Kilobase	Map Position	Result[1]
RP11-90K3	158,030	4q32	+/+
RP11-704A20	158,520	4q32	**+/-**
RP11-372N22	158,730	4q32-q33	**+/-**
RP11-27H11	160,207	4q32	**+/-**
RP11-272N13	172,998	4q33-q34	**+/-**
RP11-79K2	178,880	4q34	+/+
RP11-18D7	184,260	4q35	+/+
RP11-50L21	186,770	4q35	+/+
RP11-33M11	188,359	4q35.2	+/+

[1] +/+ Bac Clone signals on both chromosomes **+/-** Bac Clone signal deleted on one chromosome Map position of clones as of 3/23/04

Table 3: Genotyping of markers on chromosome 4q.

Marker	Father Size (bp)[1]	Mother Size (bp)	Subject Size (bp)	Distance in Kb[2]	Conclusion
Pdgfc106	148 150	150	148 150		Heterozygous
612J15	119	121 123	123		Deletion
D4S1629	146	146 150	150	158,914	Deletion
D4S2411	213 218	209	209	160,016	Deletion
D4S2982	186 194	179 198	179	161,672	Deletion
D4S2368	322	314 318	314	169,412	Deletion
D4S3028	234	224 227	227	177,286	Deletion
M6-91472	151	151 153	151 153		Heterozygous
D4S2427	300	296 300	296 300	178,449	Heterozygous
D4S1552	193	183 195	193 195	179,526	Heterozygous
D4S2924	230 232	234 236	230 236	187,148	Heterozygous

[1] basepairs [2]Kilobases Map position of polymorphic markers in NCBI as of 1/19/04

the function of the protein product is known or inferred. These contigs also contain 26 sequences that express hypothetical proteins, 9 proteins of unknown function with homology to previously identified proteins and 12 Expressed Sequence Tags (ESTs). Among the 33 genes of known or inferred function, 13 are expressed in the brain. Genes that may be considered to be candidate genes for autism include *AMPA 2 (GRIA2)* that encodes the GluR2 subunit of the AMPA glutamate receptor, *GLRA3 and GLRB* that encode glycine receptor subunits α3 and β and the Neuropeptide Y receptor encoding genes *NPY1R* and *NPY5R*.

Discussion

This study is the first case report of autism associated with an interstitial deletion on chromosome 4q. Deletion of contiguous genes in the 4q32-4q34 region could lead to the specific dysmorphic features as well as the behavioral phenotype seen in the subject. Among the deleted genes, potential candidates for autistic disorder are most likely to be those that are abundantly expressed in the brain.

Glutamate receptors are named for the pharmacological substances that influence them. AMPA glutamate receptors are responsive to alpha-amino-3-hydroxy-5-methyl-

4-isoxazole propionate (AMPA) [13]. Abraham and Bear [14] reported that AMPA receptor mediated neurotransmission plays a key role in several aspects of developmental and adult synaptic plasticity. AMPA glutamate receptors are excitatory ionotropic neurotransmitter receptors. In these the receptor itself forms an ion channel, and binding of the ligand to the receptor is followed by an influx of ions, sodium, potassium or sometimes calcium into the neuronal synapse. AMPA glutamate receptors are composed of four sub-unit types GluR1, GluR2, GluR3 and GluR4. The genes encoding these receptors are named *AMPA 1-4* (*GRIA1-4*). Each GRIA (AMPA) glutamate receptor gene encodes a protein of 900 amino acids and the different sub-units show 70% amino-acid homology. The C terminal ends of the GluR sub-units determine their interactions with other synapse associated proteins. AMPA receptors are permeable to Na+, K+ and to some degree to Ca+. GluR2 receptor sub-units are particularly involved in the control of Ca+ influx [15].

Two different splice forms of each sub-unit occur. Different splice forms predominate in various regions of the brain during adult and fetal life. There are also alterations in the ratio of GluR2 to GluR3 during development of brain. The AMPA glutamate receptors achieve high density in the cerebral cortex, hippocampus, in the basolateral and lateral nuclei of the amygdala, in the caudate, putamen, nucleus accumbens and olfactory bulb. AMPA receptors occur in the pyramidal layer of the hippocampus and in layers II and III of the cerebellar cortex. Levels of GluR2 are particularly high in the cerebellar cortex and in the Bergman glia of the cerebellum [13-15].

Receptors may be composed of identical sub-units or of different sub-units. Mansour et al. [16] reported that heteromeric receptors are made up of no more than two types of sub-units. GluR2 subunit is expressed abundantly in the hippocampal pyramidal neurons and Purkinje cells in the cerebellum [17]. Sans et al. [18] showed that in the pyramidal neurons of the hippocampus the GluR2 subunit is the major determinant of AMPA receptor structure. Furthermore at this site the GluR2 sub-unit plays a major role in receptor trafficking. Since the specific sub-units in the receptor determine the functional properties of the receptor, the relative quantities of specific receptor subunits that are available may be involved in regulating AMPA receptor function. Sans et al. [18] analyzed glutamate receptors in GluR2 knockout mice. In their studies of the hippocampus in mice with reduced or deleted GluR2 sub-units, they demonstrated that GluR2 sub-units play a critical role in the assembly and synaptic expression of the AMPA receptor complex. When GluR2 is present in sufficient amounts, GluR1-GluR3 receptor sub-types are not found. In GluR2+/- mice, GluR2 protein levels are approximately 51% of normal. In GluR2-/- mice, no GluR2 protein was present. Furthermore, in GluR2+/+ mice, Sans et al. determined that no GluR1/GluR3 receptors occurred. However, in GluR2+/- mice and in GluR2-/- mice, GluR1/Glur3 receptors were present.

Our patient is GluR2+/- and it is therefore likely that there are more GluR1/GluR3 receptors present in his hippocampus than in control subjects. It is possible that this change contributes to the pathogenesis of his autistic symptoms.

Several investigators have attributed the atypical processing of information in autism to abnormalities in hippocampal functioning. Raymond et al. [19] used the Golgi stain to analyze pyramidal cells in region CA1 and CA2 of the hippocampus. They demonstrated reduced neuronal cell size and decreased dendritic branching in brains from autistic subjects.

The brain regions that have most frequently shown anatomical changes in autism are the hippocampus and the cerebellum [20]. It is interesting to note that Purcell et al. [21] reported that the AMPA receptor density was decreased in the cerebellum of individuals with autism.

Carlsson [22] hypothesized that autism may be a hypoglutamatergic disorder based on pharmacotherapeutic studies in mice. Administration of glutamate antagonists leads to autism-like characteristics including heightened auditory and tactile perception and decreased pain sensitivity. Jamain et al. [23] reported linkage of autism to a 11 cM region on 6q21, which includes the gene that encodes the ionotropic kainite glutamate receptor subunit GluR6.

Our patient is hemizygous for the genes that encode the Glycine receptor sub-units α3 and β. Glycine and GABA are the main inhibitory transmitters in the central nervous system. Glycine acts through ionotropic receptors. In addition it modulates NMDA glutamate receptors. The glycine receptor is a glycoprotein composed of 5 subunits. Together the sub-units form glycine gated chloride receptors. The alpha sub-unit binds the glycine ligand. Each receptor is composed of three α and two β sub-units. Four different genes encode Glycine receptor α sub-units [13]. Glycine receptors occur in the spinal cord, brainstem and in the hippocampus, amygdala, striatum and cortex. Homomeric glycine receptors composed of a single type of alpha sub-unit are found in embryonic life and in the early post-natal period [24]. Glycine receptor α3 subunits are abundant in the frontal and temporal lobes and in the putamen. Two transcripts are derived from the *GLRA3* gene. One is 2.4 Kb, the other is 9 Kb in size. The precise role of glycine receptors is not understood. Okabe et al. [25] demonstrated that glycinergic membrane responses

occur early in embryonic neocortical development life in the cortical plate neurons and Cajal Retzius cells. Paton and Richter [26] and Bracci et al. [27] postulated that glycinergic inhibition governs the rhythmic output of mammalian motor systems including the medullary respiratory network. Mutations in the *GLRA1* subunit have been shown to result in hyperekplexia characterized by excessive startle reactions to unexpected, particularly auditory stimuli [28]. However, in a subset of families hyperekplexia is not associated with mutations in *GLRA1*, suggesting that other genes in the glycinergic pathway, including the α3 and β subunits of the glycine receptor, may be involved [28]. Increased sensitivity to auditory stimuli is a feature associated with autistic disorder.

There is a growing body of data that confirms that neurogenesis takes place in post-natal and in adult life. The hippocampus is one of the key sites for generation of new neurons after birth. Van Praag [29] and others demonstrated that newly generated post-natal neurons are integrated into hippocampal circuitry. Gould et al. [30] reported that hippocampal neurogenesis is enhanced by learning new skills. Collectively these studies provide evidence that post-natal neurogenesis in the hippocampus plays a role in learning and memory. Aylward et al. [31] reported that there is a reduction in the volume of amygdala and hippocampus in autistic individuals, particularly in relation to total brain volume. They concluded that the histopathology of autism suggests that these volume reductions are related to a reduction in dendritic tree and neuropil development and probably indicate the underdevelopment of the neural connections of limbic structures with other parts of the brain, particularly cerebral cortex.

The subject with autism reported here is hemizygous for genes encoding the neuropeptide Y receptors *NPY1R* and *NPY5R*. Dumont et al. [32] demonstrated that Neuropeptide Y (NPY) affects cognitive function and learning and memory. Thorsell et al. [33] reported impaired spatial learning in transgenic rats that over expressed NPY and demonstrated decreased NPY Y1 receptor binding. Michel et al. [34] reported that in the hippocampus NPY is localized to GABA-ergic interneurons and its activity is mediated through its Y1, Y2 or Y5 receptors. These receptors are coupled to G protein signaling pathways.

Howell and coworkers [35] demonstrated that NPY has a proliferative effect on hippocampal nestin positive neuronal precursor cells and on hippocampal beta tubulin positive neuroblasts. They carried out studies of the hippocampal cells in NPY Y1 receptor knockout mice. Through studies in these mice and through *in vitro* studies in the presence of selective NPY Y1 receptor agonists and antagonists, Howell et al. [35] demonstrated that the neuroproliferative effect of NPY in the hippocampus is mediated through the neuropeptide Y receptor NPY Y1 (*NPY1R*). They postulate that the effect of NPY on learning and memory may be mediated through NPY neurogenesis.

The distal breakpoint in our subject interrupts the Glycoprotein M6A gene *GPM6A*. The M6A glycoprotein was first identified in mouse brain by antibody binding assays as a factor that affects the growth of neurites in cultured cerebellar neurons [36]. The human homolog of this gene, *GPM6A* maps to chromosome 4q34 in humans and a second family member *GPM6B* maps to Xp22.2-p22.4 [37]. The human homologs of the murine M6A glycoprotein are yet to be studied thoroughly in terms of function and pathology.

Linkage studies on autism families reported by Yonan et al. [38] and Buxbaum et al. [39] provide further support for location of an autism-determining gene or genes on chromosome 4q. Yonan et al. reported a linkage peak for autism at 94 centimorgans (cM) on chromosome 4q. Buxbaum et al. reported linkage peaks between 104.9 and 126 cM on 4q. The deletion in our patient lies approximately between 157 to 177 cM.

Conclusions

This case illustrates the importance of carrying out cytogenetic and molecular genetic analysis in subjects with autism. Although there is clear evidence that genetic factors play a role in autism, many genes are likely involved. Identification of autism candidate genes through linkage analysis is hampered by the extreme genetic heterogeneity in autism. Cases of autism in which structural chromosome changes occur are invaluable since they highlight chromosome regions that are the sites of autism candidate genes.

Our molecular genetic analysis of the deletion on chromosome 4q in the patient described here highlights specific autism candidate genes. It also serves to broaden the spectrum of candidate genes to be considered for analysis in autism. Hemizygous deletion of the *AMPA 2* glutamate neurotransmitter receptor gene that encodes sub-unit GluR2 is of interest given the role of glutamate receptors in maintaining structural and functional plasticity of synapses. Hemizygosity for the neuropeptide Y receptor genes *NPY1R* and *NPY5R* is of great interest since neuropeptide Y and its receptors play a role in hippocampal learning and memory. Our patient is also hemizygous for glycine receptor encoding genes *GLRA3* and *GLRB*. Although the precise role of glycinergic receptors is not known, recent data reveal that glycinergic membrane responses occur early in embryonic neocortical development life, in the cortical plate neurons and Cajal Retzius cells.

To evaluate the significance of the genes we identified as candidates for determining the pathophysiology of autism, it will be necessary to carry out cytogenetic studies, molecular cytogenetic analyses and DNA sequence analysis in other patients with autism.

Competing interests

None declared.

Authors' contributions

SR, PF, LM – Patient interview, compilation of patient records

CM – Neuropsychiatric testing

RSE – Neurological evaluation

MB – Dysmorphology evaluation

AW – Microsatellite polymorphic marker analysis

MS, SR, MH – Preparation of BACs and molecular cytogenetic studies

SR, MS, MH, PF – Manuscript preparation

MAS – Principal investigator

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