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Autism-specific copy number variants further implicate the phosphatidylinositol signaling pathway and the glutamatergic synapse in the etiology of the disorder

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Autism spectrum disorders (ASDs) constitute a group of severe neurodevelopmental conditions with complex multifactorial etiology. In order to explore the hypothesis that submicroscopic genomic rearrangements underlie some ASD cases, we have analyzed 96 Spanish patients with idiopathic ASD after extensive clinical and laboratory screening, by array comparative genomic hybridization (aCGH) using a homemade bacterial artificial chromosome (BAC) array. Only 13 of the 238 detected copy number alterations, ranging in size from 89 kb to 2.4 Mb, were present specifically in the autistic population (12 out of 96 individuals, 12.5%). Following validation by additional molecular techniques, we have characterized these novel candidate regions containing 24 different genes including alterations in two previously reported regions of chromosome 7 associated with the ASD phenotype. Some of the genes located in ASD-specific copy number variants act in common pathways, most notably the phosphatidylinositol signaling and the glutamatergic synapse, both known to be affected in several genetic syndromes related with autism and previously associated with ASD. Our work supports the idea that the functional alteration of genes in related neuronal networks is involved in the etiology of the ASD phenotype and confirms a significant diagnostic yield for aCGH, which should probably be included in the diagnostic workup of idiopathic ASD.

INTRODUCTION

Autism spectrum disorders (ASDs) (OMIM: 209850) are a group of severe neurodevelopmental conditions, referred to a broader extent as pervasive developmental disorders, characterized by a triad associating impairments in social interactions, communication deficits and restricted repetitive and stereotyped behaviors and interests with an onset in infancy or early childhood (before 3 years). The estimated prevalence of ASD was 2–5/10 000 with a ratio four times higher in males than in females (1). In the last decades, a significant increase (6–10-fold) of prevalence has been noticed, partially explained by improvements in case ascertainment, making ASD a public health priority (2).

There is strong evidence for a genetic etiology of ASD given that the concordance rates in monozygotic twins are

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~90% for ASD, whereas concordance rates in dizygotic twins are ~10% (3). ASD is considered to have a complex multifactorial etiology involving many genes. These genetic factors would contribute to the neurobiological alterations responsible for the final array of autistic phenotypes.

ASD is found in association with other conditions in ~10% of cases, including known genetic disorders such as fragile-X syndrome, tuberous sclerosis and cytogenetic abnormalities, mostly duplications at 15q11.13 on the maternally inherited chromosome (Prader–Willi/Angelman syndrome region) and terminal deletions of chromosomes 2q or 22q (4,5). Genome-wide linkage and candidate–gene association studies have found evidence for autism susceptibility loci on more than 20 different chromosome regions including 1p, 2q, 5q, 7q, 15q, 17q 19p and Xq, although only a few of them have been replicated (6). Single-gene mutations in *SHANK3* (gene ID: 85358) (22q13.3), *NLGN3*/*NGLN4* (gene ID: 54413/57502) (Xq13/Xp22.33) and *NRXN1* genes (gene ID: 9378) (2p16) have been described as causative of ASD in a small number of patients. Additional studies and homozygosity mapping in inbred families have also revealed genes with strong susceptibility to autism, such as the neurexin family member *CNTNAP2* (gene ID: 26047) among others (7–13). Recent reports showed that submicroscopic copy number variants (CNVs) can also have a relevant role in autism (14,15) with *de novo* germline variants as a significant risk factor in sporadic forms of ASD (16). A specific CNV at 16p11.2 has been found in ~1% of the ASD patients as well as in individuals with mental retardation (17–19). In addition, the reciprocal duplication of several deletion syndromes, dup7q11.23, dup17p11.2 and dup22q11, can lead to a phenotype of mental retardation and ASD (20–22). All these data further indicate the genetic heterogeneity of ASD with different modes of inheritance and different contribution of *de novo* and inherited variants in individual families.

Given that the cause of ASD in the majority of patients is still unknown and the fact that many genomic regions have been associated with the disorder, genome-wide screening methods seem to be indicated for the identification of affected regions and specific CNVs that could be associated to the disease. We have analyzed 96 Spanish patients with idiopathic ASD after ruling out most known genetic causes, using a homemade BAC microarray for comparative genomic hybridization (aCGH) (23). With this approach, we have identified 13 novel ASD-specific copy number changes including two rearrangements in genomic regions previously associated with the ASD phenotype, such as 7q11.22 and 7q31.33–32.1. Interestingly, a significant proportion of the 24 different genes located in these 13 loci are strong functional candidates for ASD, involved in common or related neuronal signaling networks.

RESULTS

We selected for the study 96 Spanish patients (12 females and 84 males, ratio: 1/7) with idiopathic ASD after extensive clinical and laboratory screening. ASD was confirmed in all cases using the Autism Diagnostic Interview-Revised (ADI-R). All patients had been examined by neurologists and clinical geneticists and also had an extensive negative laboratory workup, including standard karyotyping, fragile X molecular testing, subtelomeric and targeted multiplex ligation probe amplification (MLPA) screening (probes for the regions 1p36, 2q37, 7q11.23, 15q11–q13, 16p11.2, 17p11.2, 22q11.2 and 22q13.3), as well as metabolic studies in some cases when clinically indicated (Gener *et al*., submitted).

Autism-specific CNVs

We detected gains and losses of a total of 238 BACs in the ASD samples with the hot-spot-BAC-array (HSBA) aCGH: 194 of these CNVs had been previously described as putatively polymorphic in the population by other authors (http://projects.tcag.ca/variation) and 18 more were also detected in our control samples ($n = 52$). We considered that two CNVs could be the same when they overlapped in >70% of their predicted genomic content. The remaining 26 variants covering 21 genomic regions (5 deletions, 15 amplifications and 1 detected as both) were present specifically in the ASD population and were then considered as possibly pathogenic (Supplementary Material, Table S1). These CNVs ranged in size from 89 kb to 2.4 Mb. On average, we detected 9.8 CNVs per individual in ASD samples and 12.4 in controls, with no significant difference.

To define the experimental variability of the hybridization signal of the BACs detecting CNVs, we performed a boxplot analysis of the $\log_2$ ratios in all control samples; 7 out of 26 BACs showed high signal dispersion, suggesting possible false-positive results (Supplementary Material, Fig. S1).

In order to validate, better define and determine whether the novel rearrangements were inherited or *de novo*, we then targeted these 21 putative pathological variants with additional molecular technologies: MLPA assays with specific synthetic probes and oligo aCGH (Agilent 44k or 244K) or SNP array (Illumina 370). Using these tools, we confirmed the CNVs in 13 regions affecting 12 patients, and we defined the size and nature of the rearrangement, being complex in some cases (Table 1, Fig. 1). A total of eight CNVs detected with the BAC array were not confirmed by the other methods, six of them corresponding to single BACs with high dispersion of the signal (Supplementary Material, Fig. 1). The MLPA assays confirmed that the rearrangements were inherited in the five patients for whom both parental samples were available. Unfortunately, samples from one or both parents of the remaining seven patients were unavailable and we could not determine whether their CNVs were *de novo* or inherited (Table 1).

We then screened our entire cohort of autistic samples ($n = 215$) and controls ($n = 120$) with a homemade MLPA panel containing probes targeting each of the CNVs found by aCGH. We did not find rearrangements in any additional patient or control individual. We also searched the database of Genomics Variants (http://projects.tcag.ca/variation) for similar rearrangements, using the data from controls ($n > 1800$) that had been analyzed by high-density oligo arrays with enough probe coverage in the regions (more than 10 probes per locus) (24–26). Although there was some partial overlap at three loci with CNVs reported in population controls (Supplementary Material, Table S1), none was of the

Table 1. Description of the confirmed CNVs affecting each of the 12 patients with ASD, including the BAC ID, chromosomal location, size, CNV type, validation methods and gene content

Sample	Gender	BAC ID	Cytoband	CNV	Validation	Start	End	Length (kb)	Origin	MLPA probe	Gene content
AUT21	F	RP11-125A7	chr13q14.11	Gain	MLPA + Illumina	41 003 320	41 418 753	415.433	MAT	KIAA0564	*KIAA0564*
AUT24	M	RP11-55L3[a] RP11-458P15 RP11-61N14 RP11-335K15[a] RP11-778G8[a] RP11-510D4[a] RP11-750O22[a] RP11-366I1[a] CTD-201L19[a] RP11-630F19[a]	chr4q26	Gain	MLPA + Agilent	117 191 242	119 614 346	2423.104	MAT	TRAM1L1	*NDST3, PRSS12, TRAM1L1*
AUT31	M	RP1-117N3	chr1p35.1	Loss	Agilent	33 158 375	33 263 299	104.924	NA		*RNF19B, AK2*
AUT42	M	RP11-140C4	chr2q33.3	Gain	MLPA + Illumina	208 759 831	208 849 748	89.917	NA	PIP5K3	*C2orf80, IDH1, PIP5K3*
AUT45	F	RP11-266G18	chr21q21.2	Gain	Illumina	25 518 184	25 708 178	189.994	NA		*No genes*
AUT84	M	RP11-510O20[a] RP11-105E3 RP11-432H11 RP11-21K15 RP11-475H14[a] CTD-210M22[a] RP11-290P11[a]	chr7q31.33–q32.1	Gain	MLPA + Agilent	125 672 999	126 255 215	582.216	NA	GRM8	*GRM8*
AUT91	M	RP11-99M10	chr18q12.1	Gain	MLPA + Illumina	10 127 382	10 594 362	466.98	NA	NAPG	*APCDD1, NAPG*
AUT96	M	RP11-21N8	chr3q21.3	Gain	MLPA + Agilent	131 845 264	131 974 345	129.081	NA	PIK3R4	*COL6A6, PIK3R4*
		RP11-575M4	chr7q11.22	Gain	MLPA + Agilent	69 630 362	69 955 721	325.359		AUTS2	*AUTS2*
AUT138	M	RP11-281H14	chr11q14.1	Loss	MLPA + Illumina	84 032 216	84 276 593	244.377	NA	DLG2	*DLG2*
AUT150	M	RP11-47L17[a] RP11-158F10 RP11-194K9	chr5q35.3	Gain	MLPA + Agilent	177 501 908	177 688 820	186.912	PAT	AGXT2L2	*COL23A1, AGXT2L2, GMCL1L, HNRNPAB, NOLA2, RMND5B*
AUT186	M	RP11-239E10	chr1q41	Loss	MLPA + Illumina	221 401 766	221 501 748	99.982	PAT	SUSD4	*SUSD4*
AUT195	M	RP11-107B3	chr3q22.3	Gain	MLPA + Illumina	139 934 042	140 070 771	136.729	PAT	PIK3CB	*PIK3CB*

The final CNV size estimate is based on oligo/SNP array data.
F, female; M, male; NA, not available; MAT, maternally inherited; PAT, paternally inherited.
[a]BACs overlapping with polymorphic CNVs described in controls.

Figure 1. Specific rearrangements detected in ASD patients. Each subfigure shows, under the patient's identifier and chromosome number, a scheme of the genomic region containing the CNV. From top to bottom: (i) chromosomal scale (Mb), (ii) G-band of the chromosome, (iii) regional CNVs described in public databases (http://projects.tcag.ca/variation), (iv) genes in the interval, (v) probes of the Illumina or Agilent arrays, (vi) MLPA probes, (vii) probes of the BAC array and (viii) plot with the results of the oligo or SNP array hybridization showing the deleted (below 0.4) or duplicated (above 0.4) intervals flanked by red dots.

size and gene content of those found in ASD patients. Thus, the CNVs found are unique in each family and very rare or absent in the general population.

More detailed phenotype data for the 12 ASD patients with specific rearrangements are shown in Table 2.

Candidate ASD genes and pathways

We considered as candidates all genes located in the ASD-unique rearranged regions that were validated with additional molecular techniques—a total of 24 genes (Supplementary Material, Table S2). Only 2 out of 24 were completely deleted (*AK2* and *RNF19B*) (gene IDs: 204/127544), 11 out of 24 completely duplicated and the remaining 11 out of 24 partially altered or disrupted.

Two of the detected alterations lie in regions of chromosome 7 previously related with ASD, 7q11.22 and 7q31.3, containing or disrupting a single candidate gene per rearrangement, *AUTS2* (gene ID: 282553) and *GRM8* (gene ID: 2918), respectively (Fig. 1). The remaining 11 CNV regions detected in ASD patients affected novel regions.

We then analyzed the data set of genes in ASD-related CNVs, using different computational resources [Ingenuity Pathway Analysis (IPA), ConsensusPathDB, KEGG], to obtain a general overview of their most relevant functions. Among the different functions encoded by those genes, the inositol and phosphatidylinositol-3-OH kinase (PI3K) signaling pathways were significantly overrepresented ($P = 1.64E + 06$) (Supplementary Material, Table S3). Interestingly, in addition to the two genes directly involved in the PI3K pathway (*PIK3CB* and *PIP5K3*) (gene IDs: 5291/200576), at least four other genes included in the ASD-specific CNVs encode proteins that act either upstream or downstream of PI3K: *PIK3R4*, *DLG2*, *AGXT2L2* and *GRM8* (Fig. 2 and Supplementary Material, Table S2), in related pathways such as the toll-like receptor signaling, the regulation of autophagy or the neuroactive ligand–receptor interaction. Therefore, at least 6 of the 13 ASD-specific CNVs affect genes with a role in related pathways, whereas only 4 of the remaining 212 non-ASD-specific CNVs contained genes in those pathways (significant *Z*-test with a 99% confidence interval). This finding strongly suggests a potential contribution of the identified ASD-specific CNVs to the ASD phenotype.

Table 2. Phenotype data of the 12 patients with autism spectrum and specific rearrangements

Patient	Sex	Diagnosis	Mental retardation	Dysmorphism	Seizures	Aggressiveness	Other features
AUT21	F	Autism	Moderate	No	No	No	
AUT24	M	Autism	Moderate	No	No	No	Neurosensorial deafness (65 dB); diaphagmatic hernia (Bochdalek)
AUT31	M	Autism	Severe	No	No	No	
AUT42	M	Autism	Severe	No	No	Yes[a]	
AUT45	F	Autism	Severe	No	Yes	Yes	Subcortical brain atrophy
AUT84	M	Autism	Severe	No	Yes	No	Hyperekplexia or startle disease
AUT91	M	Autism	Severe	No	No	No	
AUT96	M	Autism	Severe	No	No	Yes[a]	
AUT138	M	Autism	Severe	No	No	No	Hypermetropy (+5d), short stature (−2DS)
AUT150	M	Autism	Moderate	No	No	No	Obsessive-compulsive disorder
AUT186	M	PDD	Mild	Yes	No	No	Retrognatia
AUT195	M	Autism	Mild	No	No	No	Unilateral neurosensorial deafness

PDD, pervasive developmental disorder.
[a]Occasional self-injurious behavior.

To explore whether the same pathways were also overrepresented in disease-specific CNVs detected in ASD samples by other studies (15,16,27,28), we downloaded the genes in those CNVs and analyzed their functional annotation by the ConsensusPathDB. We obtained a total of 2901 genes from the available data. Using the pathway-based sets, we observed the same pathways clearly overrepresented among those genes, being on top the toll-like receptor signaling, the regulation of autophagy and the mTOR signaling ($P < 0.001$), among others (Supplementary Material, Table S4). PI3K signaling is key in the mTOR upstream activation process (Fig. 2).

Non-specific CNVs

We also analyzed the CNVs found in our ASD patients that had also been described as rare variants in controls (reported in $<1\%$ of controls) for the presence of genes belonging to the same or related pathways. Interestingly, three patients showed a gain-type CNV in the 15q13.3 region that included the *CHRNA7* gene (gene ID: 1139). Given that the flanking BACs in the array gave normal signal, this duplication likely corresponds to a $\sim$500 kb interval (29, 8–30, 3 Mb of chromosome 15) located between segmental duplications that has also been found in $\sim$1% of the general population (http://projects.tcag.ca/variation). In addition, an autistic patient showed a gain-type CNV, including the *PDPK1* gene (gene ID: 5170), and another case had a gain-type CNV, including the *RAF1* gene (gene ID: 5894). By MLPA analysis, we found that the CNVs in these five cases were inherited from unaffected progenitors. The *RASSF5* gene (gene ID: 83593) as well as five RAB family members related to the same signaling pathway were also present in non-ASD exclusive CNVs.

DISCUSSION

The use of high-throughput genomic techniques has demonstrated to be a powerful strategy for the detection of genomic abnormalities associated with ASD (15,16,27). In our hands, the use of a BAC aCGH has allowed the identification of specific regions altered in copy number in a significant proportion of our autistic population, 12 out of 96 cases (12.5%), being inherited from a normal parent in all five cases with parental samples available. Some of these regions were already reported to harbor ASD genes by either linkage and association studies or by the finding of chromosomal translocation breakpoints in ASD patients (11,29–31). Other altered regions in our autistic population that could be linked to the pathological phenotype have also been found as rare variants in relatives and/or non-autistic population. The incomplete penetrance of these rare inherited CNVs could be due to several factors including interaction with other genetic or epigenetic alterations, environmental modifiers or the unmasking of recessive alleles. This is probably the expected situation in a multifactorial and complex disease such as ASD, a group of different conditions with overlapping phenotype generated each from a single or, more frequently, a combination of genetic changes with possible environmental contribution as well. Therefore, genetic studies should search for the different multifactorial combinations.

The CNVs found in ASD patients could be merely passenger changes or have a driver contribution to the autistic phenotype should they lead to disruption or deregulation of relevant genes. We initially focused our interest in the regions that were altered only in our autistic population and had not been found in controls. We detected and confirmed 13 chromosomal alterations affecting 12 of our patients. Since we had excluded from the study ASD patients with non-idiopathic ASD after previous extensive clinical and laboratory screening with targeted MLPA analyses for subtelomeric and known rearrangements, the yield of the aCGH in detecting novel CNVs potentially related with the ASD phenotype is quite significant—12.5%. Only 3 were genomic losses, whereas 10 changes were genomic gains.

Two of the ASD-related CNVs identified map to regions of strong linkage with ASD on chromosome 7, 7q11.22 and 7q31.3, and contain genes already proposed as candidates, *AUTS2* and *GRM8*, respectively. The *AUTS2* gene, encoding

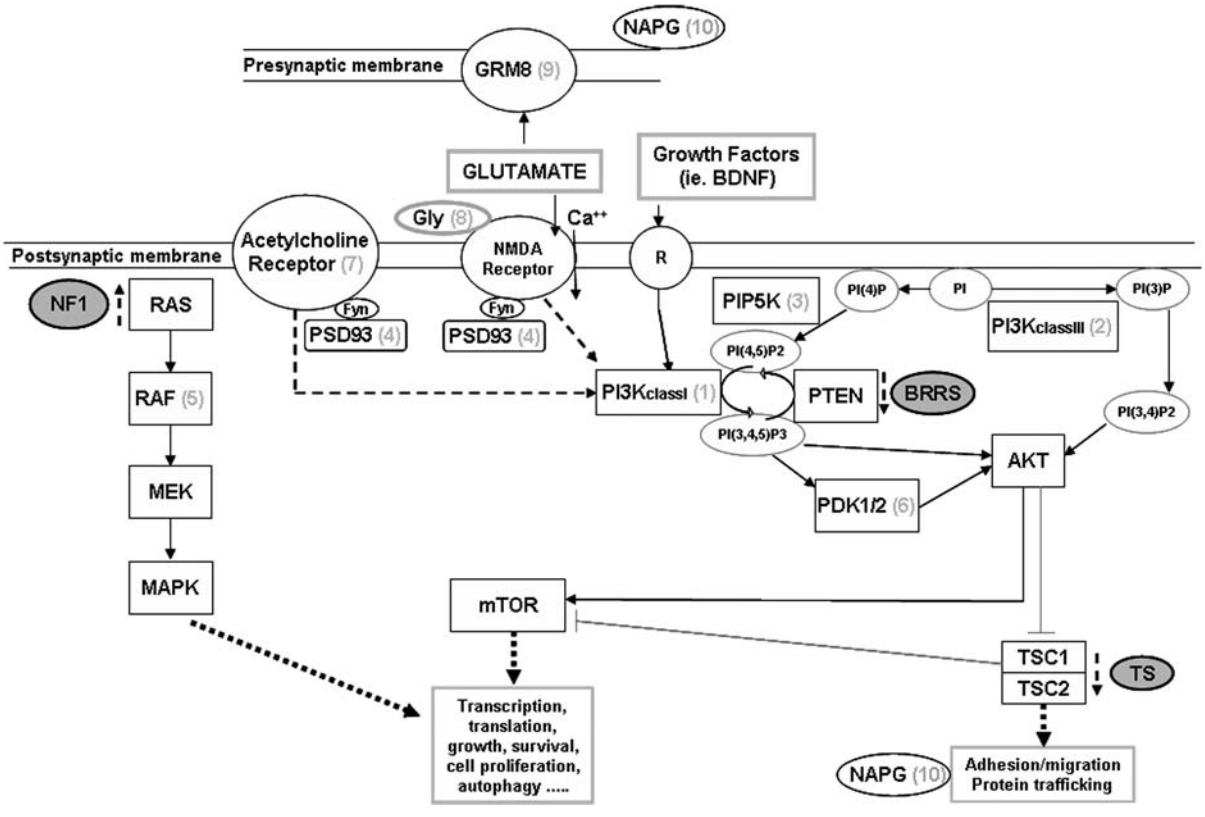

Figure 2. Schematic representation of the glutamatergic synapse and the postsynaptic neuronal signaling pathways relevant to ASD, with the putative location of the proteins encoded by the genes affected by the CNVs detected [(1) *PIK3CB*, (2) *PIK3R4*, (3) *PIP5K3*, (4) *DLG2*, (5) *RAF1*, (6) *PDPK1*, (7) *CHRNA7*, (8) *AGXT2L2*, (9) *GRM8* and (10) *NAPG*]. The interrupted arrows show the abnormal function in three monogenic disorders associated with ASD: NF1, BRRS and TSC.

a large protein of unknown function, was found disrupted at the 7q11.2 breakpoint of different balanced translocations and inversion in ASD patients, as well as in unrelated patients with severe mental retardation (11,29,30). Genetic variants in the *GRM8* gene, coding for the glutamatergic receptor 8, have also shown significant association with ASD (31). *GRM8* is a strong functional candidate given that GRM8 overactivity in the lateral amygdala leads to the inhibition of synaptic transmission impairing learned fear acquisition, a feature present in ASD (32). It has also been shown that the lateral amygdala has an abnormal growth pattern and significantly fewer neurons in autistic patients (33). Interestingly, the patient with the rearranged *GRM8* gene presented, in addition to ASD and severe mental retardation, an abnormal startle response to tactile stimuli and a diagnosis of hyperekplexia (OMIM: 149400). Dysfunction of the glutamatergic synapse in ASD is also supported by the finding of mutations in additional genes somehow involved in glutamatergic transmission, such as *NLGN3*, *NLGN4*, *NRXN1*, *SHANK3*, *CNTNAP2* and even *FMR1* (gene ID: 2332), the gene responsible for the fragile X syndrome (FXS, OMIM: 300624).

Out of the genes affected by the remaining ASD-related CNVs, we have highlighted some genes with causative potential considering their biological role. The most striking finding is that two of those genes directly participate in the PI3K signaling pathway (*PIP5K3* and *PIK3CB*) and a third gene (*PIK3R4*) is highly related. Signaling by phosphorylated species of phosphatidylinositol regulates diverse cellular processes including membrane trafficking, cytoskeletal reorganization (34) and sex-dependent synaptic patterning (35), having also a role in glutamatergic and nicotinic neurotransmission and mTOR activation (36). The putative association of this pathway with the autistic phenotype has been previously proposed by other reports (31,37,38). Transmission disequilibrium test and haplotype analyses of regions of previous linkage to autism demonstrated that polymorphisms in the *INPP1*, *PIK3CG* and *TSC2* genes (gene IDs: 3628/5294/7249), all in the PI3K pathway, are in linkage disequilibrium with autism (39).

Although the functional consequences of these three CNVs are still unknown, we propose that they all might lead to the upregulation of the PI3K pathway. The first CNV includes a partial duplication of *PIK3CB* affecting the N terminal part of the gene that contains the p85-negative regulatory subunit-binding site and the RAS-binding site but not the catalytic C terminal domain. A deletion mutant of *PIK3CB* lacking the entire p85-binding domain has been previously described that efficiently activated PI3K signaling (40). The second amplification-type CNV includes the promoter region of *PIP5K3*. It is logical to propose that overfunction might be related to ASD, since heterozygous hypomorphic mutations of this gene cause the Francois–Neetens fleck corneal dystrophy (OMIM: 121850) (41), a phenotype confined to the cornea that was not observed in our patient. A third CNV contains

a complete duplication of the *PIK3R4* gene, coding for a highly conserved protein from yeast to humans that interacts with PIK3C3 *in vivo* to regulate the protein trafficking required for PI3K activity (42).

Interestingly, we found additional genes related to the same pathway in CNVs detected in ASD patients; initially discarded because they had been reported in normal controls as rare variants. The duplication-type CNV containing the *PDPK1* gene in one patient might somehow alter the PI3K signaling pathway since PDPK1 directly phosphorylates AKT (Fig. 2). The duplication-type CNV observed in three patients at 15q13.3 containing the *CHRNA7* gene which codes for the alpha7 nicotinic acetylcholine receptor could also contribute to pathway disregulation because the cytoplasmatic signal transmission of this receptor involves PI3K (36). Microdeletion and microduplication of a larger interval in this region have recently been described as a novel syndrome with incomplete penetrance and variable phenotype including mental retardation, autistic features, epilepsy and other neuropsychiatric disorders (43).

We sought further evidence of genetic variation in the PI3K pathway in previously published data of autistic populations (15,16,27,28). Three other genes directly related to this pathway were found in ASD-associated rearrangements: five ASD patients had a loss of *GAB2* (gene ID: 9846), coding for a PI3K-negative regulator in the TCR signaling pathway, one case had a gain of *PIK3C2G* (gene ID: 5288) and one case had a *PIP5K1B* (gene ID: 8395) gene disruption by a balanced translocation (15).

Additional evidence supporting a role of the PI3K signaling pathway in autism comes from several well-characterized genetic diseases frequently comorbid with ASD, such as tuberous sclerosis (TSC, OMIM: 191100), neurofibromatosis 1 (NF1, OMIM: 162200), Bannayan–Riley–Rubalcaba syndrome (BRRS, OMIM: 153480) and FXS (44). The prevalence of autism in patients with TSC is higher than in any other condition (43–86%) (45). The TSC genes, *TSC1* and *TSC2*, code for proteins that act downstream the PI3K signaling pathway downregulating mTOR (Fig. 2). As an integrator of external stimuli, a tight regulation of mTOR activity promoting cell growth, survival and proliferation is required for neural development (46,47). BRRS patients with *PTEN* mutations may also show ASD-like phenotype. In addition, *Pten* KO mice show macrocephaly, socialization problems and exaggerated responses to sensory stimuli, along with abnormal neurons with dendritic hypertrophy (37). PTEN acts as a negative regulator of the PI3K signaling pathway, and overactivity of PI3K could overcome the PTEN-negative control (Fig. 2). NF1 has also been associated with high risk for ASD, suggesting related etiologies for both disorders (48). NF1 is due to a reduction of neurofibromin activity, a tumor-suppressor protein with RAS GTPase activity that attenuates the mitogen-activated protein kinase and PI3K pathways (49). Mice that lack neurofibromin in cortical neurons and astrocytes fail to form cortical barrels in the somatosensory cortex (50). RAS also activates the RAF1 kinase, included as a gain-type CNV in one of our ASD patients and his mother, as well as in 2 out of 506 controls (24). Finally, 33% of FXS patients fulfill criteria for ASD. FXS is caused by the silencing of *FMR1*, coding for FMRP, a negative regulator of mRNA translation. The loss of FMRP in FXS leads to mGluR-dependent LTD increase, which, at the same time, is sensitive to PI3K inhibitors (51).

The PI3K signaling pathway is one of the postsynaptic transductors of glutamate neurotransmission. The glutamatergic neurotransmission has been repeatedly implicated in some of the pathogenic mechanisms of ASD (52–54). In addition to the evidence coming from the patient with the *GRM8* alteration, another ASD case showed a disruption of the *DLG2* gene which codes for PSD93, a protein that mediates tyrosine-phosphorylation of the *N*-methyl-D-aspartic acid (NMDA) receptors by Fyn (55). This phosphorylation upregulates NMDA receptor (NMDAR) function and is also needed for its interaction with PI3K (56). Therefore, *DLG2* deletion might cause a reduction in NMDAR ability to transduce signaling through PI3K. Another putative candidate is the duplication of the *NAPG* gene coding for the *N*-ethylmaleimide-sensitive factor attachment protein gamma. NAPG interacts with syntaxin 8 and is required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus to control membrane fusion (57). Finally, the gain of *AGXT2L2* gene copy number, seen in one of our patients (AUT150), also has a potential role perturbing neurotransmission. This gene codes for a glycine biosynthesis enzyme. Glycine, in addition to its direct role as neurotransmitter, is a necessary cofactor for glutamate action through the NMDAR (58).

Local protein synthesis in neuronal dendrites is critical for synaptic plasticity. However, the signaling cascades that couple synaptic activation to dendritic protein synthesis in live neurons are not fully understood although various subtypes of glutamate receptors and the PI3K/mTOR signaling have been demonstrated to have a prevalent role in the control of synaptic activity-induced dendritic protein synthesis in hippocampal neurons. Our finding of multiple genes in these pathways affected in copy number in ASD patients further supports the hypothesis that ASD can be caused, at least in some cases, by perturbation of the regulatory mechanisms of protein synthesis in the dendrites.

The diagnosis of autism or ASD is still based on behavioral criteria that do not allow differentiating among the underlying pathologies. The finding of multiple uncommon ASD-related CNVs, each in a specific patient, further reflects the complexity and multifactorial nature of the ASD phenotype. The search for genetic and genomic variation along the whole genome is still badly needed to better ascertain the genetic background of autistic phenotypes. Given the detection yield of aCGH technologies in our and other's experience, it should probably be included in the diagnostic workup of idiopathic ASD, despite that genetic counseling may be difficult since many of the rearrangements detected can also be present in asymptomatic progenitors. In addition, more focused studies on genetic and epigenetic variations of specific pathways using the available higher resolution genomic technologies could lead to a better definition of molecular signatures, being the basis for an improved diagnosis and the ultimate development of specific therapeutic targets. The glutamatergic neurotransmission and the PI3K signaling pathway appear among the candidates for this approach.

MATERIAL AND METHODS

Patients

We have studied 96 Spanish patients (74 children followed in the neurology clinic and 22 institutionalized mentally retarded adults) with a confirmed diagnosis of one of the categories of ASD listed in the Diagnosis and Statistical Manual of Mental Diseases (DSM-IV). All patients were studied using the ADI-R to define a specific category of ASD and the Wechsler Intelligence Scale for Children or Wechsler Adult Intelligence Scale. These assessments provide a measure of general, verbal and performance IQ as well as analysis of multiple factorial components of cognitive functioning. We also use the Leiter International Performance Scale-Revised and the Raven Progressive Matrices in the non-verbal patients. All patients had an extensive evaluation by neurologists and clinical geneticists along with an intensive laboratory workup including standard karyotyping, fragile X molecular testing, subtelomeric and targeted MLPA assays (homemade panel designed to detect genomic duplications/deletions of specific regions associated with ASD and mental retardation: 1p36, 2q37, 7q11.23, 15q11–q13, 17p11.2, 16p11.2, 22q11.2 and 22q13.3), as well as metabolic and brain image studies in some cases when clinically indicated (Gener *et al*. submitted). All subjects participated after informed consent was obtained from their families or other legal caregivers. The study was approved by the medical ethical committees of the centers involved. Blood samples were obtained under institutional review board-approved informed consent, and genomic DNA was extracted by the salting out method using the Puregene® DNA Purification Kit (Gentra Systems). Parental samples were also obtained from the available parents who gave informed consent.

Controls

DNA samples from 100 population control individuals matched for population ancestry (Spanish anonymous blood donors) were used to prepare reference pools (50 males and 50 females) for hybridization experiments. DNAs from 52 control individuals (27 males and 25 females) were used in order to define polymorphic changes in DNA dosage in aCGH experiments.

CGH arrays

We used three different microarray platforms. The first screening was performed using a homemade BAC array containing 5442 large insert DNA fragments (BACs) with a global coverage of 23% of the euchromatic genome and higher probe density in genomic regions presumed hot-spots for rearrangements, named HSBA (23) (see Supplementary Material, Table S5, for more detailed information). To confirm the different variants detected with HSBA, we used two commercial arrays, either an oligoarray (Agilent 44K or 244K) or an SNP array (Illumina 370K). We performed hybridization experiments and subsequent analyses of aCGH as described previously in detail (23), and we followed the manufacturer's recommendations for the SNP array. We used the PennCNV (19 November 2008 version, sample option) and CNV partition software for the analysis of the Illumina 370K array data (59).

Multiplex ligation probe amplification

A total of 100 ng of genomic DNA from each sample was subjected to MLPA using synthetic probes designed to target the specific CNV detected by aCGH, at least one locus per CNV. Oligonucleotide sequences for MLPA at the analyzed loci are described in the Supplementary Material, Table S6. The MLPA reactions were performed essentially as described previously and products were analyzed on an ABI PRISM 3100 genetic analyzer according to manufacturers' instructions. For quantitative analysis, trace data were retrieved using the accompanying software (GeneScan, Applied Biosystems). Each MLPA signal was normalized and compared with the corresponding mean peak height obtained from five control DNA samples.

Gene ontology analyses

To obtain a general overview of the most relevant key functions represented in our data set, the genes located in the identified CNVs were used for ontology and biofunction analyses. We used the IPA software (http://www.ingenuity.com), along with two additional resources freely available: the ConsensusPathDB (http://cpdb.molgen.mpg.de) and the KEGG Pathway Database (http://www.genome.ad.jp/kegg/pathway.html) (60). We performed a core analysis to categorize the genes on the basis of their locations and their reported or suggested biochemical, biological and molecular functions. In the ConsensusPathDB, we checked the functional annotation of the gene list by using the pathway-based set options.

Statistical analysis

Fisher's exact test for count data and Pearson's χ^2 test with simulated *P*-value (based on 10 000 replicates) were applied when appropriate for measuring significant differences in gene copy number frequencies or pathway representation. In all cases, statistical significance was considered for corrected *P*-values <0.05. Differences were assessed with the χ^2 test using resampling. The *Z*-test was used in inference to determine whether the number of genes affecting relevant pathways in ASD was statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. L.A.P.-J. declares that he is founding partner and advisor of qGenomics S.L., a company involved in aCGH technology for diagnostic applications.

The other authors declare that they have no competing interests.

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