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# Screening for genomic rearrangements and methylation abnormalities of the 15q11-q13 region in autism spectrum disorders

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## Abstract

### Background

Maternally-derived duplications of the 15q11-q13 region are the most frequently reported chromosomal aberrations in autism spectrum disorders (ASD). Prader-Willi and Angelman syndromes, caused by 15q11-q13 deletions or abnormal methylation of imprinted genes, are also associated with ASD. However, the prevalence of these disorders in ASD is unknown. The aim of this study was to assess the frequency of 15q11-q13 rearrangements in a large sample of patients ascertained for ASD.

### Methods

A total of 522 patients belonging to 430 families were screened for deletions, duplications and methylation abnormalities involving 15q11-q13 using multiplex ligation-dependent probe amplification (MLPA).

### Results

We identified four patients with 15q11-q13 abnormalities: a supernumerary chromosome 15, a paternal interstitial duplication, and two subjects with Angelman syndrome, one with a maternal deletion and the other with a paternal uniparental disomy.

### Conclusions

Our results show that abnormalities of the 15q11-q13 region are a significant cause of ASD, accounting for approximately 1% of cases. Maternal interstitial 15q11-q13 duplications, previously reported to be present in 1% of patients with ASD, were not detected in our sample. Although paternal duplications of chromosome 15 remain phenotypically silent in the majority of patients, they can give rise to developmental delay and ASD in some subjects, suggesting that paternally-expressed genes in this region can contribute to ASD, albeit with reduced penetrance compared to maternal duplications. These findings indicate that patients with ASD should be routinely screened for 15q genomic imbalances and methylation abnormalities and that MLPA is a reliable, rapid and cost-effective method to perform this screening.

**MESH Keywords** Adolescent ; Adult ; Angelman Syndrome/ genetics ; Autistic Disorder/ genetics ; Child ; Child, Preschool ; Chromosome Aberrations ; Chromosomes, Human, Pair 15, genetics ; DNA Methylation/ genetics ; Female ; Gene Deletion ; Gene Dosage ; Humans ; Male ; Microsatellite Repeats/ genetics ; Prader-Willi Syndrome/ genetics ; Uniparental Disomy

**Author Keywords** autism ; chromosome 15 ; deletion ; duplication ; Angelman syndrome ; MLPA

## Introduction

Autism is a neurodevelopmental disorder characterized by impaired social interaction and communication, and a restricted range of interests and activities, with onset during the first three years of life. Autism spectrum disorders (ASDs), which include autism, pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger syndrome, have a typical male preponderance and their estimated prevalence is 6/1000 (1). ASDs are etiologically heterogeneous, with an underlying genetic disorder identified in 10%–25% of cases. Monogenic disorders such as fragile X syndrome, tuberous sclerosis, and Rett syndrome are found in a small percentage of patients; in addition, rare mutations in other genes (e.g., NLGN3, NLGN4X, PTEN, SHANK3) have been reported in a small number of individuals (2). Cytogenetically visible chromosomal aberrations are identified in 3%–6% of affected individuals (3, 4), while recent higher-resolution whole-genome analyses using array-based technologies have revealed genomic imbalances in at least 10% of cases (5–7).

Duplications of the 15q11-q13 region are the most frequently reported chromosomal aberration in individuals with ASDs (8). This region includes the Prader-Willi syndrome/Angelman syndrome (PWS/AS) critical region, which is subject to genomic imprinting. Most duplications of this interval are caused by supernumerary chromosomes formed by the inverted duplication of proximal 15q, known as isodicentric chromosome 15 [idic(15)] (9–13). Interstitial duplications of this region are less frequent (14), but many cases have been reported in association with autism (10, 13, 15–17). The majority of cases are associated with maternally-derived duplications, whereas paternal inheritance usually leads to normal phenotypes (10, 15, 16, 18).

Deletions or methylation abnormalities of chromosome 15 result in either Prader-Willi syndrome or Angelman syndrome, depending on whether they arise on the paternal or maternal chromosome. Both syndromes have been described in patients with ASD or autistic behavior, and recent studies estimate that over half of the patients with Angelman syndrome have ASD (19–21).

The genomic instability of proximal chromosome 15 is mediated by low-copy repeats, resulting in five recurrent breakpoints (BP) involved in deletions, duplications and idic(15) (22). The deletions involve either the proximal BP1 or BP2 and share the same distal breakpoint (BP3), whereas the duplications can extend more distally to BP4 or BP5 (Fig. 1). The critical region involved in PWS/AS and the 15q11-q13 duplication syndrome lies between BP2 and BP3. Nonallelic homologous recombination between flanking low-copy repeats is also involved in other microdeletion/microduplication syndromes associated with mental retardation and ASDs, including 17p11.2, 22q11.2 and 7q11.23 (23).

Although the frequency of 15q11-q13 duplications in autism is widely assumed to be 1%–3%, this estimate is based on two small series of patients. In a study of 140 subjects with autism, two were found with a maternal interstitial duplication of 15q11-q13 (24). Schroer et al. studied 100 patients with autism and identified two idic(15), one maternally-derived interstitial duplication and one maternal deletion (10). The small numbers of patients studied makes it difficult to draw conclusions on the real contribution of such duplications to ASD. The prevalence of proximal 15q deletions in ASDs is also unknown. Recent genome-wide studies of copy number variants in large ASD samples included an unknown proportion of patients who had been previously screened for chromosomal abnormalities and 15q11-q13 rearrangements (and excluded if positive), thus precluding the estimation of prevalence (5, 6).

The aim of this study was to assess the frequency of 15q11-q13 abnormalities in 522 individuals with ASDs. We screened the PWS/AS region for gene dosage alterations using multiplex ligation-dependent probe amplification (MLPA) and quantitative microsatellite analysis. In addition, we searched for methylation abnormalities of chromosome 15, including uniparental disomies and imprinting center defects, using methylation-sensitive MLPA (25). Epigenetic defects have long been suspected in autism (26, 27), but no systematic screening of methylation abnormalities of chromosome 15 had been performed.

## Methods and Materials

### Patients

A total of 522 patients with ASD belonging to 430 families were studied. Subjects were recruited by the Paris Autism Research International Sibpair (PARIS) study at specialized clinical centers in Europe and the United States. The patients included 393 males and 129 females (ratio 3:1), with a mean age at the last evaluation of 11±7.5 years (range 2.5–43); 187 belonged to 95 multiplex families (with two or more affected siblings) and 335 were sporadic cases. All patients were evaluated by psychiatrists or child neurologists and diagnosed based on clinical evaluation and DSM-IV criteria. Patients were assessed with the Autism Diagnostic Interview-Revised (ADI-R) (28) and the Asperger Syndrome Diagnostic Interview (29). Four-hundred seventy-two individuals met criteria for autism, 26 for Asperger syndrome and 24 for PDD-NOS. Three-hundred fifty-six patients (68%) had mental retardation, 261 (50%) had very limited or no language, and 66 (13%) had a history of epilepsy. Most patients were Caucasian (89%). Laboratory tests included karyotype, fragile X and metabolic screening; brain imaging and EEG were performed when possible. Patients with known genetic disorders were excluded. Chromosome analysis identified one female harboring a supernumerary isodicentric derivative chromosome 15; we report here the cytogenetic, molecular and phenotypic characterization of this patient. No other patients had been excluded from the sample prior to this

study because of cytogenetic abnormalities of chromosome 15q11-q13. The study was approved by the research ethics boards of the collaborating institutions. Informed consent was obtained from all families participating in the study.

### **Multiplex ligation-dependent probe amplification (MLPA)**

Patients were screened for rearrangements involving the 15q11-q13 region using the ME028 PWS/AS and/or the P064 MR1 MLPA kits (n=351 and 522, respectively) (MRC-Holland). The ME028 kit contains 25 probes specific for sequences in or near the PWS/AS critical region, as well as 5 probes to assess methylation status (25). The P064 kit contains 5 probes in the PWS/AS region (one probe in MKRN3, NDN, and GABRB3, and two in UBE3A). MLPA data were analyzed using GeneMarker 1.70 software (SoftGenetics). After population normalization, the peak height from each sample was compared to a synthetic control, which represents the median of all normal samples in each experiment. Peak heights below 0.75 were considered as deletions and values above 1.3 as duplications. Cases with apparent deletions or duplications were confirmed with quantitative PCR (qPCR) and fluorescent in situ hybridization (FISH). Apparent deletions of a single probe were sequenced to rule out single-base changes within the probe-binding region. For further details see Supplement 1.

### **Quantitative microsatellite analysis**

Five microsatellite markers in the 15q11-q13 region (D15S11, D15S817, D15S1513, D15S1019 and D15S815) were used to assess DNA copy number and follow the transmission of the alleles (Fig. 1). PCR conditions (in particular the number of PCR cycles) were set up so as to perform allele dosage. PCR products were quantified on an ABI3730 sequencer (Applied Biosystems).

## **Results**

Five quantitative assays using microsatellites were used to screen the 15q11-q13 region in 217 patients. However, analysis of the markers D15S11 and D15S817 revealed three alleles in 10% of patients, a proportion comparable to that in a control population, indicating that this region is duplicated in healthy individuals. We therefore decided to screen all patients using MLPA.

Overall, we identified four patients with 15q11-q13 abnormalities considered to be pathogenic: an *idic(15)*, an interstitial duplication, a deletion and an uniparental disomy. Their clinical features are summarized in Table 1 and are described in more detail in Supplement 1.

### **Supernumerary isodicentric chromosome 15**

Patient 1 had an *idic(15)* revealed by the karyotype and confirmed by FISH, described as 47,XX,+mar.ish der(15)(D15Z1++, SNRPN++, UBE3A++) (Fig. 2A). Microsatellite analysis of the patient and her parents showed that she had a *de novo* copy number gain of D15S11, D15S817 and D15S815 (Fig. 2B). Methylation-sensitive MLPA revealed that the *idic(15)* was maternally-derived, as indicated by the increased dosage of the methylated probes in SNRPN and NDN (Fig. 3). MLPA confirmed that the *idic(15)* involved all the probes in the 15q11-q13 region (between BP1 and BP3) but did not affect two genes located distally, at 15q26, BLM and IGF1R (Fig. 2C). Further analysis of the 15q11-q13 interval with 15 microsatellites showed six triallelic markers, with one allele of paternal origin and two of maternal origin (Fig. 2D). One informative microsatellite between BP4 and BP5, D15S1031, had a single maternal allele, suggesting the breakpoint lies proximal to this marker (Fig. 2D). Detailed investigation of the distal breakpoint with qPCR showed increased dosage of MTMR10, TRPM1 and KLF13, located between BP4 and BP5, but two distal genes in the same interval, OTUD7A and CHRNA7, showed a normal copy number (Fig. 2E). These findings suggest that the distal breakpoint of the *idic(15)* lies between BP4 and BP5, in agreement with the microsatellite results. The KLF13 probe showed an increase in dosage to the hexasomy range, suggesting a complex rearrangement at the site of the breakpoint. We could not differentiate between one or two breakpoints, because of the inability to estimate the precise copy number of individual probes on the basis of qPCR (Fig. 2E).

### **Interstitial 15q11-q13 duplication**

Patient 2 had three alleles for both D15S11 and D15S817. He was homozygous for D15S1513 and D15S1019, which were therefore uninformative, and heterozygous without imbalance for D15S815, located between BP3 and BP4 (not shown). MLPA confirmed the presence of a ~4.6 Mb duplication between BP2 and BP3, corresponding to the critical region deleted in PWS/AS (Fig. 4). Methylation-sensitive MLPA showed normal dosage of the methylated maternal probes in SNRPN and NDN, indicating that the duplication was paternally derived (Fig. 3). MLPA of the parents showed that the duplication was inherited from the unaffected father (Fig. 4). Methylation analysis of the father showed a paternal origin of the duplication (not shown), but no DNA was available from the grandfather to determine if the duplication was inherited or had arisen *de novo*. FISH using the UBE3A probe confirmed the presence of an increased signal in Patient 2 (not shown).

### **Angelman syndrome: deletion and uniparental disomy**

Patients 3 and 4 had only one allele for three markers located in the PWS/AS region (D15S11, D15S817 and D15S1513), suggesting that they could be either homozygous or deleted in this region (Fig. 5A). Examination of the parents' haplotypes showed that both patients

had only one allele from the father and none from the mother (Fig. 5A ). MLPA confirmed that Patient 3 had a maternal class I deletion extending ~5.6 Mb from BP1 to BP3 that arose de novo (Fig. 3 , 5C ). FISH with probe D15S10 confirmed the deletion (not shown). In Patient 4, MLPA showed a normal copy number of the 15q11-q13 region (Fig. 5C ) but methylation-sensitive MLPA revealed a complete absence of maternal methylated DNA sequences, indicating a paternal uniparental disomy (Fig. 3 ). FISH showed normal hybridization of probes SNRPN and D15S10 (not shown), consistent with the diagnosis of uniparental disomy.

### **Abnormal copy number of the BP1-BP2 region**

MLPA also detected three patients with a deletion (Patients 5, 6 and 7) and two with a duplication (Patients 8 and 9) of the BP1-BP2 interval, which spans 254 kb and encompasses four genes, TUBGCP5, CYFIP1, NIPA1, and NIPA2 (Fig. 1 ). All the rearrangements were inherited from parents who were unaffected or had unrelated psychiatric disorders (Table 1 ).

The cytoplasmic FMR1 interacting protein 1 (CYFIP1) interacts with FMRP, encoded by the fragile X mental retardation 1 (FMR1) gene. Since fragile X syndrome is often associated with autism, CYFIP1 constitutes a candidate gene for autism. In order to test the possibility that point mutations in this gene, either inherited from the other parent or de novo, could lead to autism in association with the BP1-BP2 imbalance, we sequenced the whole coding region of CYFIP1 in the five probands with abnormal dosage of this region. In Patients 5 and 8 we extended the search of point mutations to the coding regions of NIPA1, NIPA2 and TUBGCP5, as well as those of FMR1, FXR1 and FXR2, which encode proteins of the FMRP complex. No mutations were identified in any of the genes screened.

### **Other deletions or sequence variants considered non pathogenic**

We identified one patient with a paternally-inherited deletion of exon 1 in one of the transcript variants of the UBE3A gene. Because the deleted exon is noncoding and UBE3A is maternally expressed, this genomic imbalance is unlikely to have clinical consequences. In another patient, MLPA showed an apparent deletion of one probe in the SNRPN promoter/exon1 region, due to a maternally-inherited nucleotide change previously reported as a rare neutral variant (see Supplement 1 for further details on these two patients).

### **Methylation abnormalities**

Aside from the uniparental disomy identified in Patient 4, no other methylation abnormalities were observed in 331 patients screened with methylation-sensitive MLPA. In the remaining subjects, uniparental disomy was ruled out with microsatellite analysis.

## **Discussion**

In this study, we found a pathogenic rearrangement of the 15q11-q13 region in 4 out of 430 families. Thus, proximal 15q abnormalities, including deletions, supernumerary isodicentric chromosomes and interstitial duplications, are found in about 1% of patients with ASD, representing one of the most common genetic causes of ASD, together with fragile X syndrome.

### **Supernumerary chromosome 15 and ASD**

Supernumerary marker chromosomes are a relatively common cytogenetic finding, with an estimated incidence of 0.8/1000 prenatal diagnoses; those derived from chromosome 15 account for about half of all marker chromosomes (30 ). The frequency is higher in children with developmental delay, with 10 idic(15 ) identified in 2000 such cases in one study (11 ). Idic(15 ) that do not include the PWS/AS critical region have no clinical effect (31 ), whereas those including the PWS/AS region lead to a neurobehavioral phenotype including autism or autistic-like behavior, cognitive deficits, hypotonia, mild dysmorphic features, and seizures (32 ). There have been numerous reports of idic(15 ) associated with autism (9 –13 ). As in Patient 1, the vast majority of idic(15 ) that encompass the PWS/AS locus are maternally derived and arise de novo (33 ). Almost two-thirds extend beyond BP3 and the majority are asymmetrical, with two distinct breakpoints, at BP4 and BP5 (14 , 33 ). Analysis of the number of copies across the 15q11-q13 region in Patient 1 with qPCR and microsatellites predicted an atypical distal breakpoint between BP4 and BP5. A similar breakpoint was reported in a patient with an idic(15 ), between D15S1013 and D15S1031 (33 ).

Although genotype/phenotype correlation studies show that the segmental tetrasomy of idic(15 ) is associated with a more severe phenotype in terms of developmental outcome compared to interstitial triplications or duplications, indicating a dosage effect, no clear correlation has been observed between gene dosage and autism phenotype (34 ). Furthermore, no consistent relationship has been observed between rearrangement size and clinical severity (11 , 34 ).

### **Interstitial duplications and ASD**

At least 33 cases of interstitial duplications of the 15q11-q13 region have been reported in association with ASDs, according to the Autism Chromosome Rearrangement Database (<http://projects.tcag.ca/autism/> ). Interstitial duplications of proximal 15q that do not include the PWS/AS critical region have no clinical effect, are usually familial and may be considered normal variants (18 ). Patients with interstitial duplications of the PWS/AS locus have an abnormal phenotype that includes developmental delay, particularly affecting speech

and language, varying degrees of mental retardation, autism or autistic features, motor coordination difficulties, and mild or no dysmorphic features (16). The phenotype is highly variable, even among members of the same family carrying identical rearrangements (16) and can in some cases manifest as developmental language disorder and dyspraxia, without autism (35). The clinical findings in Patient 2 are similar to those described in patients with maternal 15q11-q13 duplications, although he lacks the hypotonia, motor coordination problems and joint laxity observed in other cases (16, 17).

The frequency of 15q11-q13 interstitial duplications is estimated at 1:600 individuals referred with developmental delay (36). Although previous studies in small samples of patients had suggested a frequency of 1% for maternally-derived interstitial duplications in autism (10, 24), we did not detect any such cases in our sample but found one patient harboring a paternal 15q11-q13 duplication. In agreement with our findings, Bolton et al. screened 181 patients with autism and found only one interstitial duplication of paternal origin (37). Similarly, Thomas et al. studied 327 samples referred for a suspected ASD as well as 87 patients with confirmed autistic disorder and found no 15q duplications (36). More recently, a genome-wide microarray study of 427 subjects with ASD identified two maternally-derived 15q11-q13 duplications (7). Collectively, these results suggest that maternal interstitial duplications are less frequent than previously assumed, with an estimated ~0.3% frequency (4/1369) in subjects with ASD. Based on the same studies, the estimated frequency of *idic(15)* is ~0.2% (3/1369) and that of paternal duplications ~0.1% (2/1369). Note however, that the frequency of *idic(15)* may be underestimated because previous studies included an unknown proportion of subjects that had been screened for cytogenetic abnormalities prior to inclusion. It also should be noted that none of these studies were based on epidemiological samples and the possibility of ascertainment biases cannot be excluded.

### **Maternal versus paternal duplications**

Interstitial duplications of 15q11-q13 of maternal origin are associated with developmental delay and/or autistic behavior, whereas paternally-derived duplications usually lead to a normal phenotype (10, 15, 16, 18). However, as shown in Table 2, there have been at least six reports of paternal duplications encompassing the PWS/AS region associated with an abnormal phenotype, including mental retardation, delayed or absent speech, and ASD or autistic behavior (37–41). In addition, two patients with an interstitial 15q11-q13 triplication of paternal origin have been reported (42, 43). Together with the patient with the paternal duplication described here, these findings suggest that paternally-derived duplications may lead to phenotypic effects, including autism. Thus, there may be other genetic and/or environmental/epigenetic factors operating to modify the penetrance and expressivity of paternal duplications. Similar as yet unknown factors could come into play to modify the phenotypic expression of maternal 15q11-q13 duplications, which are also associated with a wide range of developmental problems and show marked clinical variability among members of the same family, including individuals who appear unaffected (16).

It is interesting to note that several of the patients with paternally-inherited duplications or triplications described previously had Prader-Willi syndrome-like features (Table 2) (38, 40, 43), suggesting a role of paternally-expressed genes mapping within the PWS/AS region (e.g., *MKRN3*, *MAGEL2*, *NDN*, and *SNRPN-SNURF*) in the phenotypic manifestations. Several recent studies have shown that ASD is more prevalent in Prader-Willi syndrome patients than was previously thought (44), in agreement with a contribution of paternally-expressed genes to the ASD phenotype. Further research is needed to examine the effect of parental origin on phenotypic outcomes of 15q11-q13 duplications and their contribution to ASD. In particular, studies of gene expression in tissues from patients with paternal duplications may improve our understanding of the effect of these rearrangements on phenotype.

### **Angelman syndrome and ASD**

The prevalence of Angelman syndrome is estimated at 1/12,000 (45). Angelman syndrome is characterized by severe mental retardation, profound speech impairment, ataxia, and typical behavior including happy disposition with frequent laughter/smiling and hand-flapping. Seizures, microcephaly and distinctive physical traits (large open mouth, widely-spaced teeth, prognathism) are also common (46). It is caused by deficiency of the maternally-inherited *UBE3A* gene, resulting from deletion of the maternal 15q11.2-q13 region (70%), paternal uniparental disomy (2%), *UBE3A* mutations (10%), or imprinting defects (5%), with no molecular abnormality identified in the remaining patients (13).

The association between Angelman and autism has been known for more than a decade (45). According to recent data, 62% (38/61, range 50%–81%) of subjects with Angelman syndrome meet criteria for autism or ASD (19–21), suggesting that ASD may be part of the behavioral phenotype of Angelman syndrome. Although the diagnosis of ASD in Angelman syndrome is complicated by the severe intellectual disability usually observed in these patients, there is clear evidence for ASD in a subgroup of Angelman patients who exhibit social and communication deficits that are disproportionate to their overall cognitive function. There is little information about the prevalence of Angelman syndrome in ASD. Schroer et al. found one maternal 15q11-q13 deletion among 100 patients with autism (10). In this study, we identified two subjects with Angelman syndrome, one with a maternal deletion and the second with a uniparental disomy, for a ~0.5% frequency.

Because certain features of autism overlap with those seen in Angelman syndrome, including severe mental retardation, absence of speech or very limited language, epilepsy and/or abnormal EEG, and stereotyped behaviors, the diagnosis of Angelman syndrome may be overlooked in patients with ASD, as happened with our two patients. Although neither patient exhibited the classical phenotype, their clinical presentation is within the normal range of phenotypic variability observed in Angelman syndrome. In common with the majority of patients with Angelman, they exhibited severe cognitive impairment and limited or absent language, but had no ataxia or microcephaly, and seizures and unprovoked laughter (during infancy only) were observed only in one.

### **BP1-BP2 microdeletions/microduplications**

We identified three deletions and two duplications of the region between BP1 and BP2, all inherited from unaffected parents. A BP1-BP2 deletion was reported recently in a boy with mental retardation, speech delay and neurological deficits, but the interpretation was complicated by the presence of the same deletion in the father, who also showed cognitive impairment (47). Copy number gains and losses of this region have also been identified in genome-wide array analyses of control individuals (Database of Genomic Variants, <http://projects.tcag.ca/variation/>), with a population frequency estimated at 1% (48), suggesting that this may be a copy number polymorphism. In agreement with this interpretation, individuals carrying small idic(15) with extra copies of the four genes included in the BP1-BP2 interval but that do not carry extra genomic material distal to BP2 are phenotypically normal (31), suggesting that these genes do not exert clinically significant dosage effects.

Nevertheless, it is interesting to note that NIPA1, NIPA2, CYFIP1 and TUBGCP5 have been suggested to modulate the PWS/AS phenotype. Angelman patients with class I deletions (including the BP1-BP2 interval) are more likely to meet criteria for autism and lack vocalizations compared with patients having smaller class II deletions (20). Similarly, in Prader-Willi syndrome, class I deletions are associated with more obsessive-compulsive behaviors and lower intellectual ability (49), and mRNA levels of these four genes are positively correlated with better behavioral outcomes (50). More recently, BP1-BP2 deletions were shown to be significantly associated with schizophrenia (51), suggesting that these variants may increase risk for various neuropsychiatric phenotypes, albeit with low penetrance and/or variable expressivity.

One of the genes situated in this interval, CYFIP1, is particularly interesting in the context of ASD. CYFIP1 interacts with fragile X mental retardation protein (FMRP) as well as with the Rho GTPase Rac1, which plays a role in the regulation of axonal migration and dendritic spine morphology. CYFIP1 has been proposed as a potential molecular link between fragile X syndrome and 15q11-13 duplication, since both disorders result in excess free CYFIP1 (52). In order to test the possibility that the BP1-BP2 deletion unmasked a recessive mutation on the other chromosome we sequenced CYFIP1, as well as NIPA1, NIPA2, TUBGCP5, and genes encoding fragile X-related proteins (FMR1, FXR1 and FXR2), in patients carrying BP1-BP2 deletions, identifying no mutations. Clearly, more research is required to determine the phenotype correlations of BP1-BP2 deletions and duplications and the role of NIPA1, NIPA2, CYFIP1 and TUBGCP5, if any, in neurodevelopmental disorders.

## **Conclusion**

The present findings confirm that genetic abnormalities of the 15q11-q13 region are an important cause of ASD, accounting for approximately 1% of cases. Our results suggest that patients with ASD, particularly those with mental retardation, should be systematically screened for duplications, deletions and methylation abnormalities of the PWS/AS region. Routine chromosomal analysis, currently recommended in the etiological evaluation of individuals with autism, would miss many of the deletions and duplications in the 15q11-q13 region, as well as cases resulting from uniparental disomy. Traditional cytogenetic tests such as FISH are expensive and would miss some duplications and all cases of uniparental disomy. Furthermore, array-based methods are also expensive and currently are not in wide use for detecting methylation changes. Methylation-sensitive MLPA allows simple, rapid, accurate and economic screening of dosage imbalances and imprinting defects in this region, providing detailed information on the parental origin and the extent of the rearrangement, and appears thus as a method of choice to screen chromosome 15 abnormalities in patients with ASD.

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## **Footnotes:**

## **Financial Disclosures**



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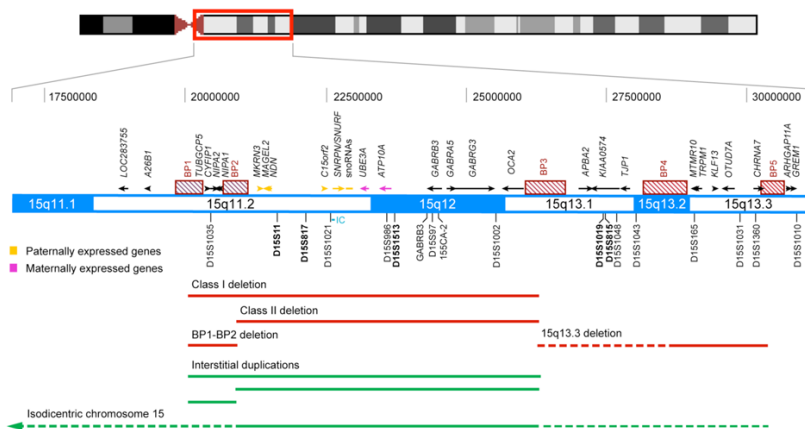
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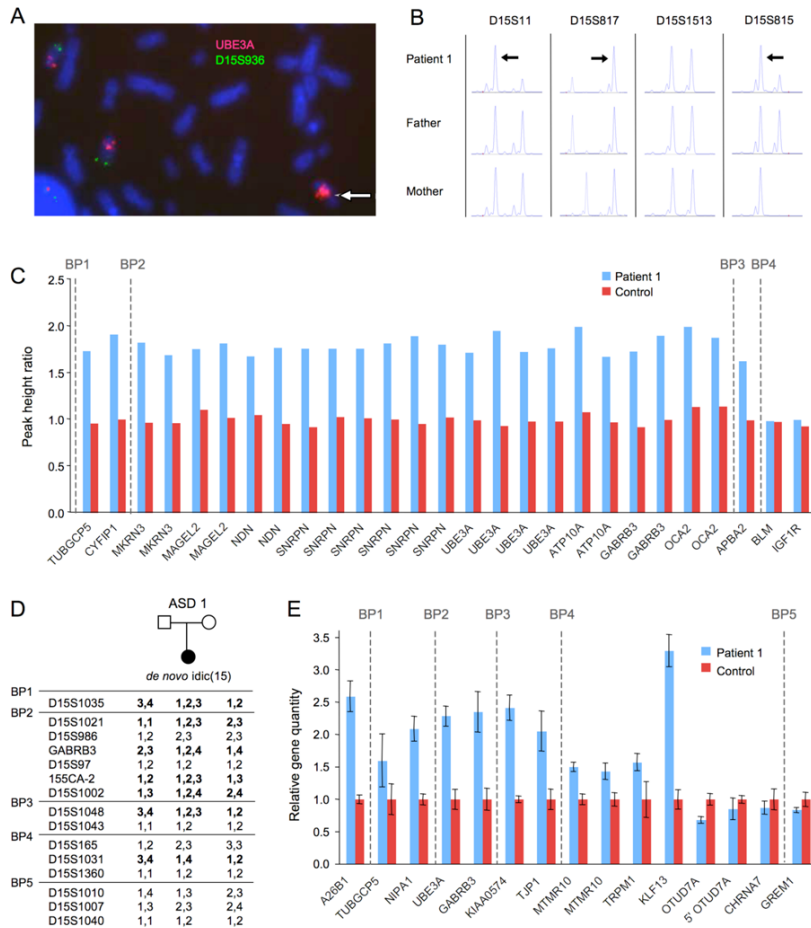
### Figure 1

Map of chromosome 15 showing the Prader-Willi/Angelman syndrome critical region. Genes are shown above the map and microsatellites below. The five microsatellite markers used in the quantitative analysis are indicated in bold. Paternally and maternally expressed genes are indicated in yellow and pink, respectively. The recurrent breakpoints (BP) are indicated as purple boxes. The imprinting center (IC), located in the 5' untranslated region of *SNRPN*, is indicated in green. The horizontal red bars indicate the regions deleted in Angelman and Prader-Willi syndromes (class I and class II deletions), as well as the recently described copy number variant between BP1 and BP2 and the 15q13.3 microdeletion syndrome. The horizontal green bars indicate the regions involved in typical interstitial duplications and supernumerary isodicentric chromosome 15. The critical region for Prader-Willi, Angelman and 15q11-q13 duplication syndromes lies between BP2 and BP3. The distance (expressed in Mb from pter) is shown at the top of the map.



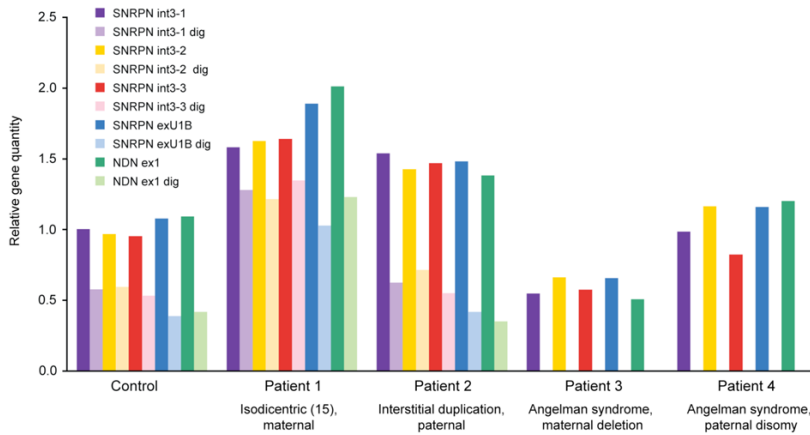
**Figure 2**

Supernumerary isodicentric chromosome 15 in Patient 1. A. Metaphase FISH of lymphoblastoid cell lines from Patient 1 using probes UBE3A (red) for the region of interest and D15S936 (green) as a control probe in 15q26.3 showed two normal signals on chromosomes 15 as well as additional hybridization spots of the duplicated 15q11-q13 region (red) on a supernumerary chromosome (arrow). B. Quantitative microsatellite analyses showed increased dosage of D15S11, D15S817 and D15S815 in the proband, but not in her parents. C. MPLA confirmed increased copy number of all the probes in the 15q11-q13 region between BP1 and BP4 (from TUBGCP5 to APBA2), and normal dosage of BLM and IGF1R, located at the distal end of the chromosome, at 15q26. The peak heights above 1.5 are consistent with tetrasomy of the region. D. Genotyping of microsatellites in the 15q11-q13 region revealed three alleles for informative markers between BP1 and BP4, with one allele of paternal origin and two of maternal origin. The presence of one informative microsatellite, D15S1031, with a single maternal allele indicates that the breakpoint lies proximal to this marker. E. Genomic qPCR showed a transition from increased copy number to normal dosage between KLF13 and OTUD7A, indicating that the distal breakpoint lies between BP4 and BP5.



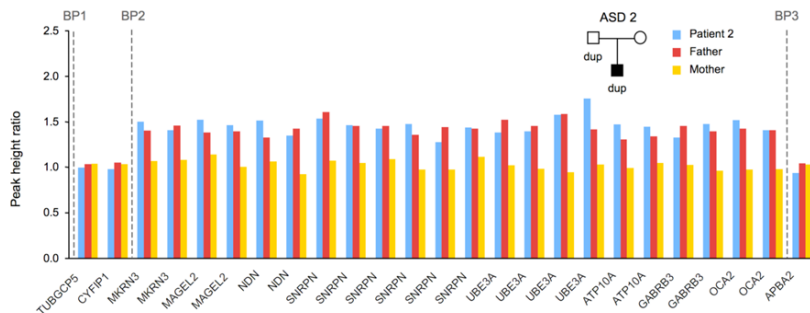
**Figure 3**

Methylation-sensitive MLPA of the chromosome 15q region. Histograms show the dosage of probes located in the SNRPN and NDN promoter regions in undigested and digested DNA. In undigested DNA, both the maternal and paternal copies of the genes are visible. Because SNRPN and NDN are paternally imprinted, after digestion with the methylation-sensitive enzyme HhaI only the maternally methylated DNA sequences are amplified, indicated by the light-colored bars. In a normal subject (control), a 50% reduction in peak height is observed after digestion. Patient 1, who has an isodicentric chromosome 15, shows 4 copies of the probes in the PWS/AS region, and a twofold increase in peak height of methylated DNA sequences, corresponding to a maternal origin of the extra chromosome. Patient 2 shows no increase in amplification of methylated DNA sequences after digestion, as expected with a duplication of paternal origin. Patient 3, with an Angelman syndrome resulting from a maternal deletion of chromosome 15q11-q13, shows a 50% reduction in peak height of probes in the PWS/AS region, and absent methylated DNA peaks after digestion. Patient 4, with Angelman syndrome resulting from a paternal uniparental disomy, shows normal peak heights before digestion with HhaI, and an absence of methylated DNA peaks afterwards, indicating the presence of two paternal chromosomes.



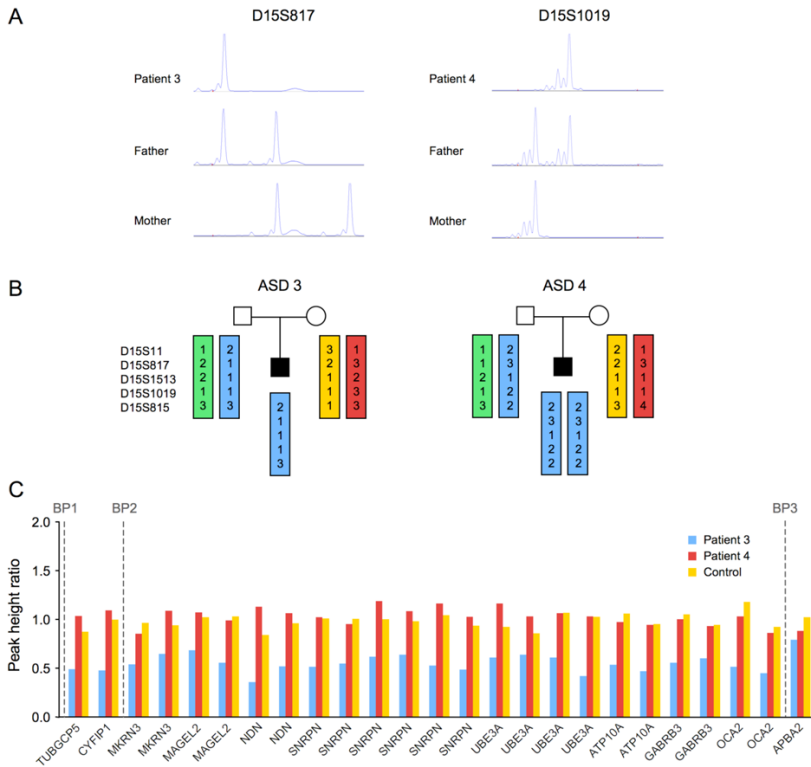
**Figure 4**

Paternally inherited interstitial duplication of chromosome 15 in family ASD 2. MPLA showed increased gene dosage of all the genes from MKRN3 to OCA2, between BP2 and BP3, while the genes before BP2 (TUBGCP5 and CYFIP1) and after BP3 (APBA2) showed normal dosage.



### Figure 5

Two patients with ASD and Angelman syndrome, one secondary to a maternal deletion (family ASD 3) and the other to a paternal uniparental disomy (family ASD 4). A. Microsatellites D15S817 and D15S1019 show loss of heterozygosity, with apparently only one allele inherited from the father in both probands. B. Genotyping of five microsatellites in the 15q11-q13 region confirmed the presence of only one allele inherited from the father in Patient 3, indicating a maternal deletion. Patient 4 showed two alleles inherited from the father, indicating a paternal uniparental disomy. C. MPLA showed normal gene dosage in Patient 4, as expected in an uniparental isodisomy, and a 50% reduction in probes ranging from TUBGCP5 to OCA2 in Patient 3, showing a class I deletion of the PWS/AS region.



**Table 1**

Clinical features of patients with rearrangements of the 15q11-q13 region

Patient	Rearrangement	Origin (chromosome)	Gender	Age at last evaluation	ASD	Developmental delay	Language	Epilepsy	Other features
<b>Pathogenic rearrangements</b>									
<b>Patient 1</b>	idic(15) (pter-15q13.3, distal BP between BP4/BP5)	de novo (maternal)	F	30 y	Autism	Severe MR	No language	Absence seizures, resistant to treatment (onset 13 y)	Feeding difficulties, psychomotor delay, dysmorphic features (large bulbous nose, dental malposition, prominent jaw), short stature
<b>Patient 2</b>	interstitial duplication (BP2-BP3)	paternal	M	6 y	Autism	Moderate MR	Speech delay, no phrases	No	Frequent otitis, mastication/swallowing difficulties, no dysmorphic features
<b>Patient 3</b>	deletion BP2-BP3 (Class I)	de novo (maternal)	M	24 y	Autism	Severe MR	No language	No	Sleep difficulties, delayed motor development, gait difficulties and inappropriate laughter during infancy, unilateral renal hypoplasia, strabismus, tongue protrusion, no dysmorphism, normal head circumference, normal brain CT, normal EEG
<b>Patient 4</b>	paternal uniparental disomy	de novo (paternal)	M	18 y	Autism	Severe MR	Limited language, no phrases	Absence seizures since early childhood; seizure-free present	Normal motor development, hyperactivity, aggressiveness, sleep difficulties, minor dysmorphic features (slightly bulbous nose, dysplastic ears, widely spaced teeth), tapering fingers, hyperextension of finger joints, bilateral genu valgum, pyramidal syndrome, slightly at spastic gait, no inappropriate laughter, normal head circumference
<b>Copy number variants likely non pathogenic</b>									
<b>Patient 5</b>	deletion BP1-BP2	paternal	M	5 y	Autism	Severe MR	No language	No (but abnormal EEG)	Hypotonia, motor delay, frequent respiratory infections during infancy, macrocephaly, retrognathia, normal brain MRI
<b>Patient 6</b>	deletion BP1-BP2	maternal	F	13 y	Autism	Normal IQ	No speech delay	No	Developmental coordination disorder, depression. The mother, who also carries the deletion, has panic disorder and depression
<b>Patient 7</b>	deletion BP1-BP2	paternal	F	9 y	Autism	Normal IQ	No speech delay	No	ADHD combined type. Two older sisters also carry the deletion, one with ADHD and the other with dyslexia, while the younger healthy brother did not inherit the deletion.
<b>Patient 8</b>	duplication BP1-BP2	maternal	M	5 y	Autism	Severe MR	Functional language	Yes (onset 9 m)	Normal brain MRI
<b>Patient 9</b>	duplication BP1-BP2	paternal	M	15 y	Autism	Severe MR	No language	1 absence seizure at 11 y	Neonatal overgrowth, macrocephaly and increased height persist, long narrow hands and feet
<b>Patient 10</b>	deletion ex 1 UBE3A	paternal	M	6 y	Autism	Mild MR	Severe language impairment	No	No dysmorphic features, normal neurological exam

ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BP, breakpoint; CT, computed tomography; EEG, electroencephalogram; idic(15), isodicentric chromosome 15; MR, mental retardation; MRI, magnetic resonance imaging

**Table 2**

Patients with paternally-derived interstitial duplications or triplications of chromosome 15q11-q13 with an abnormal phenotype reported in the literature

Study	Rearrangement	Sex	Age	Clinical characteristics
Mohandas et al., 1999 <sup>(39)</sup>	duplication (at BP2-BP3)	at least M	2 y	developmental delay, absent speech, partial agenesis of the corpus callosum and heterotopic gray matter in the right hemisphere; no dysmorphic features
Engelen et al., 1999 <sup>(38)</sup>	duplication (at BP2-BP3)	at least M	12 y	features similar to Prader-Willi syndrome, including developmental delay, obesity starting during the first year of life, mild mental retardation, excessive eating, skin picking, minor dysmorphic features (high forehead, upslanting palpebral fissures), myopia, and small hands
Mao et al., 2000 <sup>(40)</sup>	duplication (at BP2-BP3)	at least M	16 y	developmental and speech delay, behavioral problems (mood liability, social immaturity), uncontrolled appetite, food stealing, self-injurious behavior, depression and anxiety, low average IQ, short stature, obesity, no dysmorphic features
Roberts et al., 2002, Bolton et al., 2004, Veltman et al., 2005 <sup>(37, 53, 54)</sup>	duplication BP1-BP3	F	5 y	proband: motor and speech delay, PDD-NOS, borderline mental retardation, clumsy and uncoordinated, stiff gait, mild hypotonia, joint laxity, slight down-slanting palpebral fissures
	duplication BP1-BP3	F	8 y	older sister: language and speech delay, developmental motor coordination disorder, oppositional defiant behavior, encopresis, autistic behavior when younger but no ASD at 8 y, low average IQ; clumsy, mild hypotonia, joint laxity*
Smith et al., 2004 <sup>(41)</sup>	duplication BP2-BP3	F	17 y	hypotonia, development and speech delay, autism, dyscalculia, short stature, minimal dysmorphism and motor coordination problems; normal IQ
Cassidy et al., 1996 <sup>(42)</sup>	triplication BP2-BP3 (BP1-2 not studied) (BP3-4 normal)	F	6 y	neonatal hypotonia, difficulty feeding, motor and speech delay (words 2.5 y, phrases 6 y), violent and repetitive behavior, high pain tolerance, short stature, wide mouth, normal neurological exam
Ungaro et al., 2001 <sup>(43)</sup>	triplication BP1-BP4	F	12 y	features similar to Prader-Willi syndrome, including mild mental retardation, cleft palate, obesity, compulsive eating, small hands and feet, and short stature

All rearrangements included the Prader-Willi syndrome/Angelman syndrome critical region. All duplications/triplications were characterized molecularly and parental transmission was assessed with methylation studies, except Engelen et al.<sup>(38)</sup> who determined parental origin based on cytogenetic polymorphisms, and Smith et al.<sup>(41)</sup>, who used microsatellite analysis. In addition, Engelen et al.<sup>(38)</sup> observed the duplication with FISH but microsatellite analysis showed no duplication and the methylation pattern was normal, thus making this case questionable.

\* Another sibling, who did not carry the duplication, also had mild hypotonia and a history of marked speech delay and articulatory dyspraxia, while the mother had delayed speech and troubles with reading and writing in school.

Abbreviations: ASD, autism spectrum disorder; BP, breakpoint, PDD-NOS, pervasive developmental disorder not otherwise specified