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ARTICLE

Identification of novel deletions of 15q11q13 in Angelman syndrome by array-CGH: molecular characterization and genotype–phenotype correlations

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Angelman syndrome (AS) is a neurodevelopmental disorder characterized by mental retardation, absent speech, ataxia, and a happy disposition. Deletions of the 15q11q13 region are found in approximately 70% of AS patients. The deletions are sub-classified into class I and class II based on their sizes of ~6.8 and ~6.0, respectively, with two different proximal breakpoints and a common distal breakpoint. Utilizing a chromosome 15-specific comparative genomic hybridization genomic microarray (array-CGH), we have identified, determined the deletion sizes, and mapped the breakpoints in a cohort of 44 cases, to relate those breakpoints to the genomic architecture and derive more precise genotype–phenotype correlations. Interestingly four patients of the 44 studied (9.1%) had novel and unusually large deletions, and are reported here. This is the first report of very large deletions of 15q11q13 resulting in AS; the largest deletion being >10.6 Mb. These novel deletions involve three different distal breakpoints, two of which have been earlier shown to be involved in the generation of isodicentric 15q chromosomes (idic15). Additionally, precise determination of the deletion breakpoints reveals the presence of directly oriented low-copy repeats (LCRs) flanking the recurrent and novel breakpoints. The LCRs are adequate in size, orientation, and homology to enable abnormal recombination events leading to deletions and duplications. This genomic organization provides evidence for a common mechanism for the generation of both common and rare deletion types. Larger deletions result in a loss of several genes outside the common Angelman syndrome–Prader–Willi syndrome (AS–PWS) critical interval, and a more severe phenotype.
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Introduction

Genomic rearrangements affecting the proximal segment of human chromosome 15 result in a number of well-defined clinical phenotypes.[1,2] This region of chromosome 15 (15q11q13) is extremely complex in its organization and susceptibility to rearrangements. Angelman syndrome (AS) and Prader–Willi syndrome (PWS) are disorders

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mapping to 15q11q13, a region that includes a >4 Mb domain subject to genomic imprinting.[3–5] Several different chromosomal abnormalities involving 15q11q13 have also been identified in cases of autism often with a dependence on parent of origin.[6–8] AS (MIM 105830) is a neurodevelopmental disorder characterized by mental retardation, absent speech, dysmorphic features, ataxia, seizures, and typical behavioral abnormalities, including a happy sociable disposition.[9] AS is caused by deficiency of maternally inherited *UBE3A* (E6-associated protein ubiquitin-protein ligase gene), located within the imprinted region on chromosome 15q11q13.[10] Deletions of the 15q11q13 region account for approximately 70% of the AS patients. Three common AS/PWS chromosomal breakpoints (proximal BP1, BP2, and a common distal BP3) are involved in a majority of recurrent deletion/duplication events involving 15q11q13 and the existence of large low-copy repeats (LCRs) in the vicinity of these breakpoints has been documented.[5,11–13] We previously showed that children with larger, class I deletions were significantly more likely to meet criteria for autism, and had lower cognitive and expressive language scores compared with children having smaller class II deletions. Children with class I deletions also required more medications to control their seizures in comparison with the class II group.[14]

We report here a detailed characterization of unusually large deletions in four patients with AS using microarray-based comparative genomic hybridization (array-CGH) and precisely mapped the deletion breakpoints and the genomic structure/sequence around them. Cognitive, behavioral, and developmental assessments were performed and genotype–phenotype correlations are reported.

Materials and methods

Clinical evaluations

The four cases described in this manuscript represent a subset of 44 deletion bearing AS patients studied previously (reported in part earlier) (three males and one female; age range 20 months to 3 years).[14] The following clinical evaluations were performed to facilitate genotype–phenotype correlations: head circumference; developmental assessments using standardized testing for cognitive skills, language, and adaptive behavior; formal evaluations for autism; evaluation of seizures including quality, frequency and drug requirements for control; and assessment of EEG studies where available (16 channel EEG recorded awake and asleep).

All patients received clinical evaluations from a psychologist. Psychologists were blinded to a child's deletion class when conducting clinical evaluations. The Bayley Scales of Infant Development, Second Edition (BSID-II) were used to assess cognitive and motor skills.[15] Parents were interviewed using the standardized administration of the Vineland Adaptive Behavior Scales – Interview Edition (VABS).[16] The Preschool Language Scale, Third Edition (PLS-III) was used to assess communication skills.[17] Two of the participants were also given the Autism Diagnostic Observation Schedule – Generic, Module 1 (ADOS).[18]

Molecular studies

Microarray-based CGH: an enhanced version of our chromosome 15-specific genomic microarray was utilized for comparative genomic hybridization. This array included a total of 175 genomic BAC (bacterial artificial chromosome) clones across the length of the long arm of chromosome 15, with the highest density of clones spanning the ~10 Mb 15q11–q14 interval encompassing the PWS/AS critical region. Additionally, 160 clones (BACs and P1-derived artificial chromosomes) specific for the subtelomeric regions of all other chromosomes were included. The validation of genomic clones, and production and analysis of array-CGH experiments were carried out as described previously.[8] The breakpoints defining each deletion predicted by array CGH were confirmed by FISH analysis, utilizing clones within and flanking the deletion.

Results

Clinical profiles and preliminary molecular analysis

Initial evaluation at first diagnosis of AS included FISH analysis for deletions involving the 15q11q13 AS/PWS critical interval. Deletion-positive individuals were subjected to further clinical evaluation and molecular analysis by array-CGH to define size and nature of 15q11q13 deletions.

Case 1: This male child was diagnosed with AS at 9 months of age when a cytogenetically visible deletion of chromosome 15q11q13 was found and confirmed by FISH. He had severe peripheral and truncal hypotonia that persisted until nearly 4 years of age. He had significant feeding difficulties, and gastroesophageal reflux disease with esophagitis and ulcers. Seizures were first diagnosed at age 26 months when a myoclonic seizure was observed during a neurology visit. In retrospect, seizures started several months earlier and were both myoclonic and drop seizures. The seizures are generally well controlled by clonazepam, but he still experiences drop attacks every few weeks. His eye contact is poor.

Case 2: This male child had severe hypotonia and feeding difficulties as an infant. Feeding problems initially improved, but then he developed an aversion to solid foods and seemed to be in pain when eating. He was diagnosed with AS at age 7 months and started to have clonic seizures before his first birthday. He was initially treated with phenobarbital and valproic acid, but changed to topiramate and high doses of valproic acid due to breakthrough seizures. He was treated with ranitidine, but feeding difficulties persisted, and endoscopy showed

Table 1 Psychological testing of patients 1–4

	Deletion size (Mb)	*Chronological age Months*	*Bayley mental age equiv. Months*	*Bayley motor age equiv. Months*	*Preschool language auditory comprehension age equiv. Months*	*Preschool language expressive comm. age equiv. Months*	*Preschool language composite age equiv. Months*	*Vineland communication age equiv. Months*	*Vineland daily living age equiv. Months*	*Vineland socialization age equiv. Months*
Patient 1	8.9	45	8	8	3	5	5	9	12	9
Patient 2	9.4	26	1	2	2	3	2	4	8	3
Patient 3	7.1	77	7	7	6	3	4	9	12	7
Patient 4	10.68	27	8	7	8	3	6	6	13	10
Mean scores for class I deletion patients			10.2	11.9	7.8	3.89	6.71	11.5	15.4	11
Mean scores for class II deletion patients			12.1	12.5	8.5	6.00	8.53	13.5	16.7	15

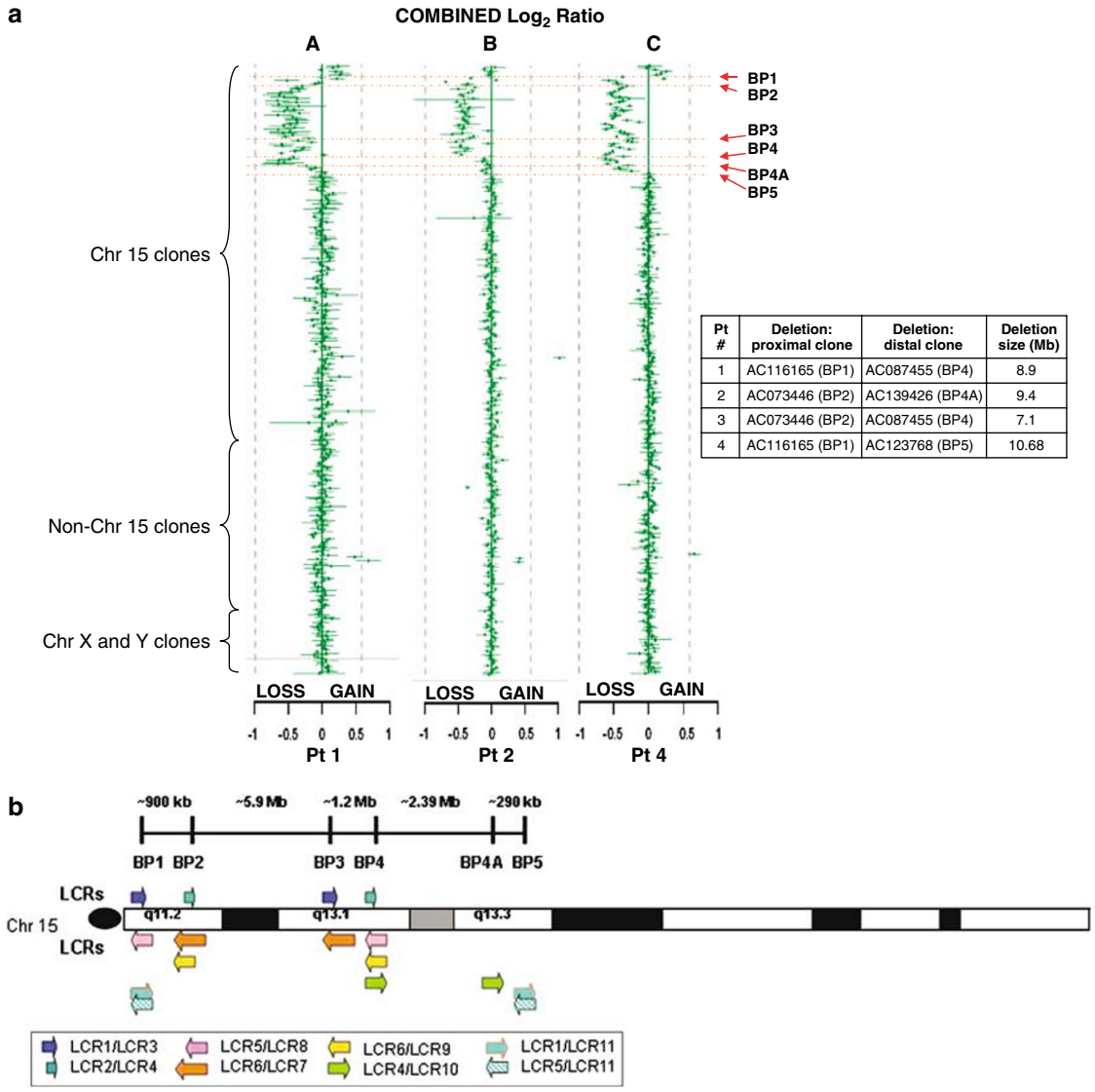
chronic esophagitis. He was changed to omeprazole and domperidone. At 26 months of age, he is microcephalic, has poor auditory attention, and exhibits sensory defensiveness.

Case 3: This male child was diagnosed with AS at age 18 months when he presented with new onset seizures. His medical history is significant for temperature instability early on in life even though he was born full term. He also has a history of severe hypotonia, global developmental delay, failure to thrive, poor feeding, weak suck, and swallowing difficulties. He was diagnosed with gastroesophageal reflux and started on Zantac. His last EEG showed typical findings for AS, with moderate to high voltage at 3–5 Hz at the fronto-central regions and intermittent high and extremely high voltage, very slow (delta) activity recorded in the frontal regions bilaterally. There was no occipital dominant rhythm and the background activity was diffusely slow. There were scattered spike and wave discharges representing potential epiloptegenic findings. He is currently treated with valproic acid and clonazepam for his seizures.

Case 4: This female child diagnosed with AS at age 7 months following a history of hypotonia from birth, global developmental delay, and a seizure disorder that started at age 5 months. Her karyotype analysis showed a visible deletion of an interstitial band at 15q11 and her methylation pattern was consistent with AS. Her EEG showed predominantly bifrontal high-amplitude spike sharp waves and sharp slow complexes, with high-amplitude bifrontal and bioccipital delta activity consistent with epilepsy. Her medical history is significant for a large patent ductus arteriosus (PDA) that required surgical intervention, a grade IV right side vesico-ureteric reflux with low implantation of the ureter, and mild kyphosis and scoliosis apparently secondary to her hypotonia. Her occipital-fronto circumference was normal at her last examination. She has had an increase in the number of absence seizures, which have been treated with escalating doses of topimarate and clonazepam, despite which she continues to make slow development progress.

Results of psychological testing

Table 1 below compares the results of the developmental testing in patients with novel deletions with their peers having typical class I and class II deletions. No formal statistical analysis is possible due to the small number of cases. In examining scores from developmental evaluations, it is clear, however, that all four patients had a more severe phenotype. All scored lower than the overall mean scores for children with class I deletions and for children with class II deletions for: their cognitive abilities, motor skills, parental (Vineland) reports of their communication, self-help skills, and socialization skills. On language testing, patients 1 and 2 achieved lower scores for their receptive language skills as compared with patients with class I and class II deletions. Patients 3 and 4 achieved receptive language scores that were consistent with patients with class I deletions but were still lower as compared with patients with class II deletions. Patients 2–4 achieved lower expressive language scores as compared with their peers. Patient 1 achieved scores for expressive language that were higher as compared with most individuals with class I deletions. Broadly, the overall language abilities of the four patients with larger deletions were also lower as compared with patients with class I and / or class II deletions. Patients 3 and 4 were formally evaluated using the Autism Diagnostic Observation Schedule, Module 1, and both were found to meet formal criteria for autism. Patient 2 had significant motor impairments; his withdrawal from the study precluded formal autism evaluation. On clinical evaluations, however, he was noted to exhibit several characteristics of



Pt #	Deletion: proximal clone	Deletion: distal clone	Deletion size (Mb)
1	AC116165 (BP1)	AC087455 (BP4)	8.9
2	AC073446 (BP2)	AC139426 (BP4A)	9.4
3	AC073446 (BP2)	AC087455 (BP4)	7.1
4	AC116165 (BP1)	AC123768 (BP5)	10.68

Figure 1 Array-CGH results and segmental duplication content at common and novel breakpoints. (**a**) Combined $\log_2$ ratio plots of array hybridization results for three AS deletion cases hybridized against normal male DNA. The plot is clone-by-clone order starting from 15 centromere to 15q telomere, followed by chromosomes 1 to Y (vertical axis: top to bottom). The locations of the common deletion/duplication breakpoints (BP1, BP2, BP3, and BP4) are indicated by arrows alongside the plot. Novel deletion breakpoints are denoted 4A and 5. Plot A, patient 1: BP1→BP4 loss (~8.9 Mb); plot B, Patient 2: BP2→BP4A loss (~9.4 Mb); plot C, patient 4: BP1→BP5 loss (~10.68 Mb). Tabular insert shows deletion size and proximal and distal clones within deleted segment. (**b**) Position and size of most relevant LCRs within or flanking breakpoints are shown with reference to the breakpoints (BP1–BP5). The LCRS are colored according to homology and orientation and described further in 'Results' section. LCRs with complete or significant homology are colored identically. The approximate size of each segment between any two breakpoints is shown along top bar (not to scale). Positions of the most significant LCRs are as follows (size in parentheses): LCR1: 20.20–20.30 Mb (103 kb), LCR2: 20.85–21.06 Mb (19–213 kb), LCR3: 26.50–26.72 Mb (106 kb), LCR4: 28.16–28.22 Mb (72–300 kb), LCR5: 20.22–20.3 Mb (72–79 kb), LCR6: 20.84–21.00 Mb (203 kb), LCR7: 26.56–26.77 Mb (214 kb), LCR8: 28.16–28.47 Mb (19.7–41.57 kb), LCR9: 28.47–28.7 Mb (300 kb), LCR10: 30.23–30.5 Mb (307 kb), LCR11: 30.47–30.7 Mb (218 kb).

children with autism spectrum disorders including: a lack of a responsive social smile, difficulties with engaging him to complete activities, lack of eye gaze, lack of shared enjoyment in interactions, a preference for playing alone and spending considerable time engaging in hand-wringing or sucking his hands. He also had food/texture aversions and did not like to wear clothing. Using DSM-IV criteria, he would meet formal criteria for autism. Patient 1 did not return to complete a formal evaluation for autism using the ADOS. Based on observations from clinical evaluations, he would not meet DSM-IV criteria for autism, but would fulfill criteria for an autism spectrum disorder (pervasive developmental disorder, not otherwise specified).

All four patients had seizures (drop attacks, partial complex seizures, and generalized seizures). Only one of the four was treated with a single anti-convulsant medication; the other three required a second medication for adequate seizure control. Three of the four children presented with significant feeding difficulties and/or gastrointestinal abnormalities (reflux, swallowing disorders, and texture aversion).

Deletion sizes and breakpoints

Previous studies from this group and others have delineated the common deletions and duplications involving the proximal long arm of chromosome 15. We and others have identified the segments flanking the common breakpoints.[8,19] The common deletions spanning the segment between BP1 or BP2 to BP3 have been estimated from recent studies to include about 6 Mb. The four probands reported herein harbor larger deletions extending telomeric beyond BP3 (Figure 1a; Table 1). The breakpoints involved in these large deletions are less common distal breakpoints (BP4, BP4A, and BP5), and common proximal breakpoints (either BP1 or BP2). Patient #2 has a deletion (BP2-BP4A) encompassing clone AC073446 to AC139426 and encompassing a segment of ~9.4 Mb. Breakpoint 4A is immediately proximal to the more common BP5. Details of the segmental losses are provided as part of table within Figure 1a. Patient #3 was briefly described in an earlier report[8] and more extensive phenotypic details and characterization of deletion is provided in this manuscript. Patient #4 represents a case with the largest deletion detected so far and with a severe Angelman phenotype. Interestingly, the distal breakpoints BP4 and BP5 have been shown earlier to be involved in the generation of interstitial 15q11q13 duplications and in formation of isodicentric 15(q).[8,19]

Genomic structure across breakpoints

An accurate positioning of the recurrent and novel deletion breakpoints provides us an opportunity to investigate the genomic structure across the boundaries for mechanistic clues. Interestingly, the common as well as the new breakpoints are all flanked by large segmental duplications (intrachromosomal low-copy repeats, LCRs). Earlier evidence implicated the presence of multiple copies of a common repeat that is composed primarily of a large transcript (*HERC2*) and distributed across all the common breakpoints. Identification of these uncommon deletions and their associated breakpoints reveals the presence of intrachromosomal LCRs flanking every breakpoint. The most significant LCRs across these common and rare deletion breakpoints are depicted in Figure 1b. BP I is flanked by a ~103.6 kb LCR1 encompassing the nucleotide positions 20.2–20.3 Mb, with homology to directly oriented LCR at BP3 (LCR3, size ~106 kb). Two highly homologous LCRs (LCR6 at 20.84–21 Mb and LCR7 at 26.56–26.77 Mb respectively) flanking BP2 and BP3 and oriented opposite to LCRs 2 and 3 represent the LCRs most likely responsible for the recurrent class II deletions (BP2–BP3). LCRs 8 and 9 are two oppositely oriented LCRs that overlap with LCR4 and are homologous to LCRs 5 and 6, respectively, at BP1 and BP2. There are embedded within these two LCRs, multiple small LCRs in both orientations of 15.1–19.5 kb in size that are homologous to similar segments at BP1 and BP2 (Figure 1b); size and degree of homology is less than that seen for BP1–BP3 and BP2–BP3 flanking LCRs that might explain the rarity of these unusual deletions. Additionally LCR9 retains some homology to the more distal LCR10 at BP4A. The involvement of these two LCRs in deletion/duplication events may not be a common event, but may potentially present a risk factor for submicroscopic rearrangements of 15q13q14. LCR11 (at 30.47 Mb; ~218 kb in size) at BP5 reveals segments of homology and directly oriented with the LCRs 1, and 5 respectively, though the overlap is considerably less than seen for the more commonly recombinogenic LCRs (eg, LCRs 1, 2, 3, and 4). This combination of direct repeats possibly reflects a role in BP1–BP5 and BP2–BP4A deletions identified in this series.

The *HERC2* duplication with a primary location at nucleotides 26.03 to 26.240×10^6 is also represented by multiple homologous duplicons of various sizes that localize to regions flanking BP1, BP2, BP3, and also to a segment at 16p11.2.[11,13,20–22] BLAT analysis of the *HERC2* gene (15.3 kb mRNA; position: chr15:26029785–26240890; genomic size: 211106) reveals significant stretches of homology primarily across BP2 (LCR2/LCR6), BP3 (LCR3/LCR7), and to a lesser extent to regions in the vicinity of BP1 (LCR1). Additionally, there are homologies to segments centromeric to BP1 at the 18.89–18.98 Mb position. The *HERC2*-related duplicons therefore share considerable sequence homology with the LCRs described above and most likely share a common evolution.

Discussion

Phenotypic characterization of AS patients with accurate delineation of the molecular defect provides the opportunity to determine genotype–phenotype relationships. Newer genomic array-based technologies have helped to define the nature of the common deletions of 15q11q13 resulting in AS and to identify novel deletion rearrangements of proximal 15q resulting in AS. Earlier studies by our group and others have shown that children with AS, who had larger class I deletions, were significantly more impaired cognitively and behaviorally. Significantly, there is a trend for class I individuals to have lower expressive and total language abilities and an increased likelihood of meeting criteria for a comorbid diagnosis of autism. Atypical deletions involving 15q11q13 resulting in AS are

rare and have not been characterized previously due to the lack of high-resolution tools for accurate molecular karyotyping. The genomic clones flanking the common breakpoints involved in duplications, isodicentric 15q, and the typical class I and class II deletions have been characterized earlier,[8,19] with agreement from independent studies utilizing newer genomic array-CGH-based tools. These studies define the common deletions to be approximately ~6 Mb in size and involve one of two proximal breakpoints (BP1 or BP2) and a common distal breakpoint (BP3). This estimate is more accurate and substantially larger than earlier estimates using conventional molecular and cytogenetic tools.[8] The current study reveals the presence of rare, previously unreported, deletions with distal breakpoints telomeric to the common BP3. Interestingly, these novel distal breakpoints (BP4, BP4A, and BP5) are all flanked by large intrachromosomal LCRs, the same genomic structure that mediates the more common class I and class II deletions. Characterizing these breakpoints provides a more unifying mechanism for the genesis of deletions (common and uncommon) of 15q11q13. These LCRs are large and are primarily intrachromosomal, except one LCR with homology to a segment on 16p11.2. The body of evidence supports a common mechanism for these deletions and duplications. This is likely to involve meiotic misalignment and illegitimate homologous recombination between LCRs. As is shown in the data presented and *in silico* analysis of the genomic structure across these breakpoints, LCRs positioned in the immediate vicinity of these breakpoints are appropriately oriented (direct repeats and homologous oppositely oriented repeats) and of adequate size so as to recombine against each other and result in deletion events. Perfect homology between oppositely oriented LCRs at the same breakpoints provides considerable evidence in support of identical LCR-mediated recombination-based mechanism for the generation of interstitial duplications/isodicentric chromosome involving the same 15q11q13 segment.

Earlier molecular and cytogenetic analyses demonstrated multiple duplicated segments of copies of a large gene, *HERC2*, localized in and around the recurrent breakpoints.[11,13] Present analysis with the availability of complete sequence makes it clear that LCRs other than *HERC2* repeats comprise an important component of the genomic structure spanning 15q11q13. In contrast to interstitial 15q11q13 deletions that have BP3 as the common distal breakpoint, isodicentric chromosome 15s are generated with involvement of more distal breakpoints (BP4, BP5). It is possible that smaller isodicentric 15s involving BP3 occur but are lost during cell division and hence rarely identified cytogenetically. Nevertheless, a clearer picture of the mechanism is revealed from these analyses of the genomic structure at the deletion breakpoints. As one could have predicted, larger deletions would be mechanistically possible due to the extensive similarity in the LCRs at multiple locations within 15q11q13. Identification of these novel deletion carrying patients confirms that theoretical prediction. Larger deletions probably do occur, but might not be compatible with life and hence remain elusive in postnatal samples. It can be speculated that the extent of homology between LCRs might be a reasonable explanation for the different frequencies of the different deletions, the more homologous the LCRs, the higher the frequency of illegitimate recombination and resulting deletions.

These larger deletions include a number of genes (*APBA2*, *TJP1*, *TRPM1*, and *CHRNA7*) that could impact the phenotypic outcome in these children. The *APBA2* protein is a member of the X11 protein family and functions as a neuronal adaptor protein that in turn binds to and stabilizes the Alzheimer disease amyloid precursor protein (APP) preventing its proteolytic cleavage. It is also believed to be involved in signal transduction and to act as a putative vesicular trafficking protein in the brain that can form a complex with the potential to couple synaptic vesicle exocytosis to neuronal cell adhesion.[23,24] Tight junction (zonula occludens) protein 1 (TJP1), also referred to as ZO-1, is a 200 kDa protein located on a cytoplasmic membrane surface of vertebrate intercellular tight junctions. Although the function of *TJP1* is unknown, the cDNA sequence predicted a multidomain signaling protein homologous to the product of the 'discs large-1' (*DLG*) tumor suppressor gene of *Drosophila* and several other membrane-associated proteins in mammals. Members of the DLG family of proteins are implicated in X-linked mental retardation and 3q29 deletion syndrome, autism being a significant phenotypic component in both.[25] The *TRPM1* gene (transient receptor potential cation channel subfamily M 1) is similar to the transient receptor potential (Trp) calcium channel family members. The expression of this protein is inversely correlated with melanoma aggressiveness, suggesting that it is involved in suppression of melanoma metastasis.[26] The *CHRNA7* gene is a member of the neuronal nicotinic acetylcholine receptor (nAChRs) superfamily of ligand-gated ion channels that mediate fast signal transmission at synapses. The protein encoded by this gene forms a homo-oligomeric channel, displays marked permeability to calcium ions, and is a major component of brain nicotinic receptors that are blocked by, and highly sensitive to, alpha-bungarotoxin. Binding of this channel protein by acetylcholine leads to a change in conformation and opening of an ion conducting channel across the plasma membrane. This gene has been implicated as a major susceptibility locus for juvenile myoclonic epilepsy and a chromosomal location involved in the genetic transmission of schizophrenia. An evolutionarily recent partial duplication event in this region results in a hybrid containing sequence from this gene and a novel *FAM7A* gene. Additionally, perturbations of nicotinic receptors, reduced gene expression of the alpha4/beta2 nicotinic receptor in the cerebral cortex and post-transcrip-

tional abnormalities of both this and the alpha7 receptor in the cerebellum, are an important feature of the neurochemical pathology of autism.[27–30]

It is not surprising that patients having larger deletions in the 15q11q13 region up to 10.68 Mb in size, were expected to exhibit a more severe phenotype. This has been confirmed by our clinical investigations and highlighted in Table 1 showing lower scores across all areas of mental development, socialization, and language. We therefore believe that not all deletions in the 15q11q13 region are created equally, and that similar studies dissecting the molecular and clinical phenotype will be extremely important to understand the differences presented among AS deletion patients.

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