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The clinical significance of small copy number variants in neurodevelopmental disorders

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ABSTRACT

Background Despite abundant evidence for pathogenicity of large copy number variants (CNVs) in neurodevelopmental disorders (NDDs), the individual significance of genome-wide rare CNVs <500 kb has not been well elucidated in a clinical context.

Methods By high-resolution chromosomal microarray analysis, we investigated the clinical significance of all rare non-polymorphic exonic CNVs sizing 1–500 kb in a cohort of 714 patients with undiagnosed NDDs.

Results We detected 96 rare CNVs <500 kb affecting coding regions, of which 58 (60.4%) were confirmed. 6 of 14 confirmed de novo, one of two homozygous and four heterozygous inherited CNVs affected the known microdeletion regions 17q21.31, 16p11.2 and 2p21 or OMIM morbid genes (*CASK, CREBBP, PAFAH1B1, SATB2; AUTS2*, *NRXN3*, *GRM8*). Two further de novo CNVs affecting single genes (*MED13L*, *CTNND2*) were instrumental in delineating novel recurrent conditions. For the first time, we here report exonic deletions of *CTNND2* causing low normal IQ with learning difficulties with or without autism spectrum disorder. Additionally, we discovered a homozygous out-of-frame deletion of *ACOT7* associated with features comparable to the published mouse model. In total, 24.1% of the confirmed small CNVs were categorised as pathogenic or likely pathogenic (median size 130 kb), 17.2% as likely benign, 3.4% represented incidental findings and 55.2% remained unclear.

Conclusions These results verify the diagnostic relevance of genome-wide rare CNVs <500 kb, which were found pathogenic in ∼2% (14/714) of cases (1.1% de novo, 0.3% homozygous, 0.6% inherited) and highlight their inherent potential for discovery of new conditions.

INTRODUCTION

Neurodevelopmental disorders (NDDs) are a group of conditions characterised by cognitive, neurological and/or psychiatric manifestations occurring during the development of the nervous system.[1] The various clinical entities such as intellectual disability (ID), speech and language delay, autism, neuromotor dysfunction or epilepsy show considerable comorbidity and may be associated with a variety of non-neurological features within complex syndromes.[2 3] Due to the extensive aetiological heterogeneity of NDDs, the majority of patients remain without aetiological diagnosis, which hampers disease management, genetic counselling and in-depth studies of the underlying molecular mechanisms. With the advent of new genomic technologies, however, diagnostic yield is steadily improving and a rapidly growing number of novel, aetiologically defined disorders are delineated.

Genome-wide chromosomal microarray analysis (CMA) for detection of copy number variants (CNVs) is currently used as a first-tier diagnostic approach in patients with idiopathic NDDs. The diagnostic yield of clinically significant CNVs varies between 5% and 20%, depending on the clinical preselection and resolution of the array.[4] Despite their obvious higher sensitivity, the widespread use of high-resolution arrays, however, is hampered by their inherent burden of detecting polymorphic or unclear variants. Indeed, tiling array studies have revealed a huge diversity of CNVs in the general population with an overall median length of about 2.9 kb and 95% being less than 100 kb.[5] Furthermore, CNVs larger than 500 kb were shown to occur only in about 10% of control individuals, while patients with NDDs harbour an additional burden of more than 13.5% for such CNVs.[6] Accordingly, a 2010 consensus statement on diagnostic chromosomal microarray testing recommends a resolution of ≥400 kb throughout the genome as a balance of analytical and clinical sensitivity.[4] Therefore, the individual significance of rare small CNVs has not been well elucidated in a clinical context, but is of rising interest given the recent progress in detection of small CNVs from whole-exome sequencing (WES) data.[7–9]

Therefore, in this study, we investigated the diagnostic relevance and inherent potential for gene discovery of rare CNVs sizing 1–500 kb in a cohort of 714 patients with isolated or syndromic NDDs.

METHODS

Excluding patients with large-scale chromosomal aberrations, CNVs >10 Mb, or clinically recognised recurrent microdeletion syndromes, we investigated 714 patients with NDDs with or without further congenital anomalies by genome-wide high-resolution CMA. The vast majority of patients were of European origin. Among them, 63 patients (8.8%) had obvious pathogenic CNVs >500 kb with a median size of 3.8 Mb.

We investigated CNVs sizing 1–500 kb for their overlap with annotated exons as well as with in-house and public control databases. CNVs affecting exonic regions that were not observed in our in-house controls or only reported once in public databases were tested by multiplex ligation-dependent probe amplification (MLPA) or fluorescence in situ hybridization (FISH) if not already confirmed by inheritance pattern from trio microarray analysis. Confirmed CNVs were individually assessed regarding literature evidence for pathogenicity, overlapping CNVs in the DECIPHER database,[10] function and expression profiles of the affected gene(s) and inheritance pattern. Selected candidate genes within inherited rare CNVs were further studied by Sanger sequencing for biallelic mutations. Five patients were further investigated for non-allelic hits by WES. Four of these patients were selected for WES because they had de novo CNVs affecting good candidate genes but lacking overlapping cases at the time of analysis, and one patient was exome sequenced because an inherited variant was present in three affected siblings.

Microarray and confirmatory studies

DNA, extracted from peripheral blood, was analysed with Affymetrix Genome-Wide Human SNP Array 6.0 (1.8 million markers; 79 patients), Affymetrix Cytogenetics 2.7 (2.7 million markers; 423 patients) and CytoScan HD (2.6 million markers; 212 patients) (Affymetrix Inc., Santa Clara, California, USA). The average intermarker spacing was 1.6 kb for the 6.0 array and about 1.1 kb for the two other arrays. CNVs were called if they encompassed at least five consecutive markers resulting in a maximum resolution of about 2 kb. The data set of each patient's sample was evaluated with Affymetrix Chromosome Analysis Suite (ChAS V.1.0.1) in comparison with 670 controls in the 6.0 array, 820 controls in the 2.7 array and 1038 controls in the CytoScan array. Controls consisted of European and American healthy individuals. Categorisation of CNVs by the Affymetrix's ChAS software among others includes confidence values for the 2.7 and CytoScan arrays. Confidence is determined on a marker by marker basis by evaluating the concordance of the log2ratio at each marker with the copy number state assigned by the hidden Markov model (HMM). The average confidence score of markers in gain and loss segments determines the confidence score of that segment.

Readily available kits or customised MLPA was performed using synthetic probes for selected exons and the SALSA MLPA kit P300 Human DNA reference-2 (MRC-Holland, Amsterdam, The Netherlands). The MLPA module of the Sequence Pilot 3.5.2 Build 508 software (JSI medical systems GmbH, Kippenheim, Germany) was used to retrieve relative peak intensities by normalisation to the reference probe set. Normalised peak levels were set in relation to at least three healthy control individuals. FISH analyses were performed using locus-specific commercial probes according to standard protocols on metaphase preparations from peripheral blood.

SATB2 protein modelling

The protein was modelled with Modeller 9.9,[11] based on the crystal structure of the homologous SATB1 tetramer [12] that exhibits 78% sequence identity.

Exome sequencing and mutation analysis

WES on genomic DNA of selected patients was performed as described before with minor modifications.[13] [14] All exons and flanking intronic nucleotides of candidate genes from CMA or candidate nucleotide variants from WES were analysed after PCR amplification from patient's DNA by Sanger sequencing using an ABI Genetic Analyzer 3730 (Applied Biosystems, Foster City, California, USA).

Expression studies of *ACOT7*

Expression levels were investigated in cDNA panels from fetal and adult human tissues using customised SYBR green qPCR for exons 1 and 2 of ACOT7 (specific for isoform ENST00000377855). Relative expression levels normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were set into relation to the mean expression value of this isoform in fetal brain.

Statistical analysis

Statistical differences for size, number of markers/size and confidence value of CNVs were analysed using Mann-Whitney U-testing and independent-samples t testing. p Values less than 0.05 were considered statistically significant.

RESULTS

Among the 714 array results, 96 aberrations below 500 kb fulfilled the abovementioned criteria and were further evaluated (size range 2–492 kb, median 72 kb). In total, 58 out of 96 (60.4%) of these selected CNVs were confirmed by secondary testing, while 38 of 96 (39.6%) were not confirmed and thus considered false positive (see online supplementary table S1). False positive aberrations were significantly smaller in size (3–181 kb, median 19 kb, mean 45.3 kb) than true CNVs (2–492 kb, median 131 kb, mean 164.7 kb) ($p<0.0001$; figure 1A). There was also a significant difference between the two groups regarding their confidence values (mean of 88.9% vs 91.9%, $p<0.0001$; figure 1B) and marker count (median of 20 (8–142) vs 96 (6–1040), mean of 39.9 vs 163.8, $p<0.0001$; figure 1C), while no significant difference was observed for the marker count per kb within the CNV (1.5±1.14 vs 1.1±0.67, $p=0.1$; figure 1D). Since the 1:2 copy number reduction in deletions is more easily detectable than the 3:2 copy number gain in duplications, sensitivity and specificity is different for deletions and duplications. Duplications sizing at least 183 kb or encompassing at least 168 markers were all true positives, while deletions were reliable if they sized at least 113 kb or encompassed at least 52 markers.

Among 58 confirmed CNVs, 14 (24.1%) were de novo (table 1), 2 (3.4%) were homozygous and 39 (67.2%) were heterozygous and inherited (19 from mothers and 20 from fathers). While for 12 of 14 de novo CNVs both parents were available for testing, in another two CNVs de novo origin was assumed based on their well-established causal involvement in severe and fully penetrant phenotypes (one exonic deletion within the *PAFAH1B1* gene causing lissencephaly type 1 and the recurrent 473 kb microdeletion in 17q21.31) (table 1). For two further CNVs (3.4%), patterns of inheritance could not be completely tested because the fathers were not available, but due to familial recurrence they were considered likely inherited (see online supplementary table S2). One incidental finding (*DMD* deletion) was not tested in the mother.

De novo CNVs

Six de novo or likely de novo CNVs were clearly pathogenic affecting the recurrent microdeletion region in 17q21.31

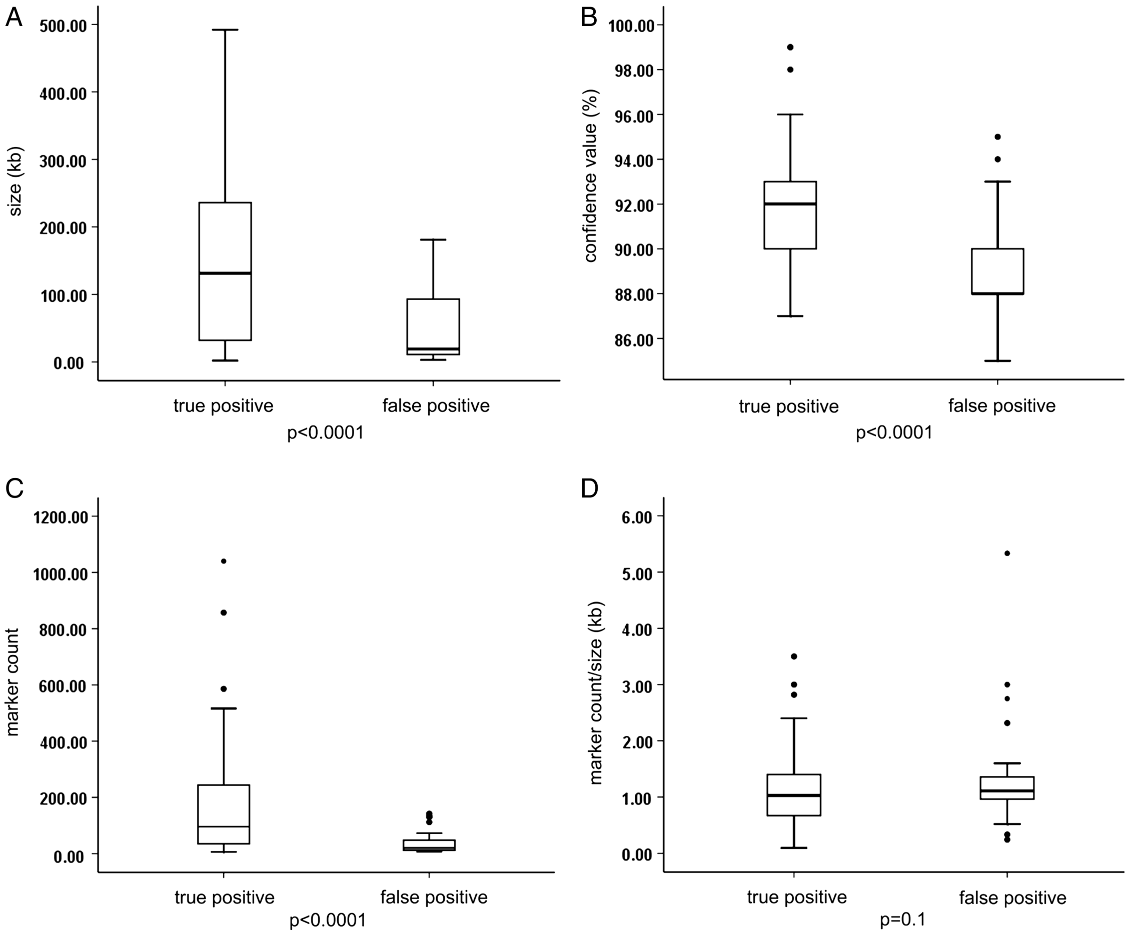

Figure 1 Comparison between true versus false positive status of small copy number variants (CNVs) detected by chromosomal microarray analysis (CMA) and their size, confidence value, marker count and marker count per kb. (A) False positive CNVs were significantly smaller in size (3–181 kb, median 19 kb, mean 45.3 kb) than true CNVs (2–492 kb, median 131 kb, mean 164.7 kb) ($p<0.0001$). (B) There was also a significant difference between the two groups regarding their confidence values (mean of 88.9% vs 91.9%, $p<0.0001$) and (C) marker count (median of 20 vs 96, mean of 39.9 vs 163.8, $p<0.0001$). (D) No significant difference was observed for the marker count per kb within the CNV (1.5±1.14 vs 1.1±0.67, $p=0.1$).

(detected in two cases), well-characterised OMIM morbid genes (*CASK*, *CREBBP*, *PAFAH1B1*) or the recently described *SATB2* locus (table 1). For two further de novo CNVs affecting single genes (*MED13L*, *CTNND2*), similar cases were identified in the DECIPHER database (http://decipher.sanger.ac.uk/). The 17 kb out-of-frame de novo deletion encompassing exon 2 of *MED13L* (MIM *608771) in patient 56366, together with overlapping cases, were instrumental to define a recognisable haploinsufficiency syndrome that we reported and discussed in detail elsewhere.[13] The novel condition caused by *CTNND2* haploinsufficiency is described for the first time below. We also report a special tooth phenotype found in our patient with *SATB2* defect. Additionally, we discuss a de novo variant limited to the *DNM3* gene, which is the candidate critical gene in 1q24-q25 deletions, as well as two de novo CNVs classified as likely benign after identification of pathogenic mutations by WES.

Novel *CTNND2*-related phenotype defined by patient 62563 with 113 kb deletion and overlapping cases

CTNND2 (MIM *604275) encodes δ-catenin, which functions as a regulator of neuronal migration[15] and maintenance of dendrites and dendritic spines in mature cortex.[16] It was mapped to the cri-du-chat syndrome critical region in chromosome 5p15.2 and was considered responsible for severe ID in typical cri-du-chat syndrome patients with terminal 5p deletions.[17] However, extended deletion mapping indicated that interstitial deletions restricted to the ID critical region 2 (MRII) including the *CTNND2* locus produce a milder level of intellectual impairment.[18] CNVs encompassing *CTNND2* have been implicated in autism (one deletion, de novo),[19] cerebral palsy (one duplication including the first exon of *CTNND2*, maternally inherited)[20] and schizophrenia (one duplication affecting seven genes including *CTNND2*).[21]

CMA in our patient revealed a 113 kb de novo out-of-frame deletion encompassing exons 4–7 of *CTNND2*. Sanger sequencing in the patient did not reveal an additional pathogenic point mutation of the gene. The girl was born spontaneously at term with normal measurements and no complication to highly educated unrelated parents. She had no remarkable health issues and developmental milestones and growth parameters were within normal limits (table 2). Physical examination revealed deep set eyes, prominent cheeks, narrow eyebrows, short inner canthal distance (ICD 2.7 cm, 2nd centile), low-set, slightly backwards rotated ears, and a bulbous nose with prominent columella. She had mild clinodactyly of the fifth finger, which was also present in the healthy brother. She was referred to formal developmental testing at age 8 years because of behavioural issues and was diagnosed with borderline ID (WISC-IV, full scale IQ 77). She showed a dissociated cognitive profile with better language (vocabulary, comprehension, reading) than non-verbal functions (visual perception,

Table 1 Clinical and genetic features of patients with candidate de novo CNVs <500 kb sorted by descending size

Patient ID	Age* (years)	Gender	Phenotype	Aberration	Chromosome band	Genome coordinates	Size (kb)	Confidence value (%)	Marker count	Affected gene(s)	Validation	Pathogenicity
52253	5	M	Developmental delay, marked hypotonia, agenesis of corpus callosum and facial dysmorphic features	Deletion (heterozygous)	17q21.31	hg18, chr17: 41049320-41522088	473	N/A	329	9 genes including *MAPT*	MLPA/-(parents N/A, likely de novo)	Pathogenic (recurrent microdeletion syndrome)[58]
69234	2	F	Developmental delay, hypotonia, microcephaly and minor morphological abnormalities	Deletion (heterozygous)	Xp11.4	hg19, chrX: 41339667-41811516	472	91	1040	*CASK*, *GPR34* and *GPR82*	MLPA/de novo	Pathogenic (phenotypic spectrum associated with CASK loss of function has been described)[59]
71264	2	M	Developmental delay, hypotonia, mild facial dysmorphic features and stridor	Deletion (heterozygous)	17q21.31	hg19, chr17: 43703800-44163085	459	90	857	9 genes including *MAPT*	MLPA/de novo	Pathogenic (recurrent microdeletion syndrome)[58]
59248	4	F	Developmental delay, severe hypotonia since birth, refractory epilepsy, facial dysmorphic features and oedematous hands and feet with tapering fingers	Deletion (heterozygous)	1q24.3	hg18, chr1: 170135864-170505998	372	N/A	244	*DNM3*	FISH/de novo	VOUS (WES of the patient and both parents did not reveal any obvious candidate gene mutation. *DNM3* encodes dynamin 3, involved in vesicular transport)
71156	1	F	Developmental delay, microcephaly and facial dysmorphic features	Deletion (heterozygous)	16p13.3	hg19, chr16: 3788867- 3935836	147	91	353	*CREBBP*	MLPA/de novo	Pathogenic (OMIM gene for Rubinstein–Taybi syndrome)[48]
62848	5	F	Developmental delay, hyperactivity and microcephaly	Duplication (heterozygous)	12q24.23	hg18, chr12: 117061815- 117183853	122	N/A	71	*TAOK3* and *PEBP1*	MLPA/de novo	Likely benign (pathogenic heterozygous mutation in *SHANK2*: c.2669_2670insC (p. P891Sfs*32) was found in this patient by WES)
62563	11	F	Learning difficulties, short attention, deficits in social-emotional behaviour and mild facial dysmorphic features	Deletion (heterozygous)	5p15.2	hg18, chr5: 11431816- 11545236	113	N/A	99	*CTNND2*	MLPA/de novo	Pathogenic (implicated in the ID phenotype of cri-du-chat syndrome.[17] Further patients are discussed in this paper)
72125	7	F	Developmental delay, and mild facial dysmorphic features	Duplication (heterozygous)	10p14	hg19, chr10: 7932363-8033508	101	90	108	*TAF3*	MLPA/de novo	VOUS (*TAF3* encodes TAF3 RNA polymerase II, TATA box binding protein-associated factor)
70229	3	M	Global developmental delay with prominent speech delay, truncal ataxia, agenesis of corpus callosum and repaired cleft palate	Deletion (heterozygous)	12q24.33	hg19, chr12: 132552537-132623611	71	90	48	*EP400*, *EP400NL*, and *DDX51*	MLPA/de novo	VOUS (there is patient 262376 in Decipher database with a duplication encompassing the same genes)
43552	19	M	Intellectual disability (ID), speech problems, spastic movement disorder and tall stature	Deletion (heterozygous)	16p13.3	hg18, chr16: 4986264- 5046682	60	89	53	*NAGPA*, *C16orf89*, and *SEC14L5*	MLPA/de novo	VOUS (a smaller deletion including only *SEC14L5* and *NAGPA* is present in 1/1038 of a world-wide control cohort by Affymetrix)
70886	4	M			2q33.1		32	89	68	*SATB2*	MLPA/de novo	

Continued

Table 1 Continued

Patient ID	Age* (years)	Gender	Phenotype	Aberration	Chromosome band	Genome coordinates	Size (kb)	Confidence value (%)	Marker count	Affected gene(s)	Validation	Pathogenicity
			Developmental delay, a doubled row of upper incisors and cleft palate	Duplication (heterozygous)		hg19, chr2: 200278502- 200310272						Pathogenic (deletions of *SATB2* have been implicated as causative for cleft palate and ID)[26]
45333	9	M	Severe ID, hypotonia, macrocephaly, haemangioma of the upper lip, bilateral postaxial foot polydactyly and obesity	Deletion (heterozygous)	20q13.32	hg19, chr20: 57556968- 57575495	19	94	28	*TH1L* and *CTSZ*	MLPA/de novo	Likely benign (pathogenic mosaic heterozygous mutation in *PIK3CA*: c.2740G>A (p. G914R) was found in this patient by WES)[34]
56366	4.5	F	Developmental delay, hypotonia, gross and fine motor coordination problems, facial dysmorphic features, and complex congenital heart defect (details reported elsewhere)[13]	Deletion (heterozygous)	12q24.21	hg18, chr12: 115158648- 115175505	17	87	18	*MED13L*	MLPA/de novo	Pathogenic (described elsewhere)[13]
617	24	F	ID, epilepsy, loss of motor function, lissencephaly type I	Deletion (heterozygous)	17p13.3	hg18, chr17: 2519114- 2523280	4	96	6	*PAFAH1B1 (LIS1)*	MLPA/-(parents N/A, likely de novo)	Pathogenic (OMIM gene for lissencephaly)[60]

*Age at the time of array.
CNV, copy number variant; N/A, not available; VOUS, variant of uncertain significance; WES, whole-exome sequencing.

abstract reasoning). She also showed short attention span, poor executive functioning and impaired working memory. The cognitive profile remained stable at follow-up. Although immature social-emotional behaviour was described, formal signs of autism were not present.

We found three further patients with exonic deletions limited to *CTNND2* via the DECIPHER database (table 2, figure 2). These three deletions were inherited and for two transmitting parents low normal IQ was recognised. Because exonic *CTNND2* deletions are not reported in the normal population databases and since these patients as well as two of the three transmitting parents share borderline low IQ or mild ID with or without autistic behavioural problems, we assume that *CTNND2* haploinsufficiency is causing the neurodevelopmental features in these patients. Given the mild phenotype of the patients, transmission by seemingly normal parents may be explained by clinical variability or lack of formal cognitive testing. The progressive neurological signs in patient 4 (DECIPHER 271234), however, may be caused by an additional unidentified disorder.

SATB2 intragenic 32 kb duplication in patient 70886 with ID and double row of upper incisors

SATB2 (special AT-rich sequence binding protein 2; MIM *608148) is a DNA-binding protein that regulates gene expression and corticocortical connections in the developing cerebral cortex and craniofacial patterning.[22 23] De novo interrupting translocations, microdeletions and mutations of *SATB2* have been described in patients with ID, behavioural problems, seizures and craniofacial anomalies with or without cleft palate.[14 24–28] The duplication detected by CMA in our patient affects exon 4 (c.170-?_346+?; p.Gly57_Gln115dup) (ENST00000457245) and is predicted by homology modelling to disturb protein tetramerisation, which plays an important role for long-range chromatin organisation and coordination in gene regulation (figure 3A).[12]

This patient was a 4-year-old boy born at term with normal measurements (51 cm; 3330 g) to healthy unrelated parents. After birth hypotonia, feeding difficulties and a cleft soft palate were noted and at age 1 year a double row of upper incisors became evident (figure 3B). At age 3 3/12 years, he was remarkably hypotonic and had borderline microcephaly (OFC 49 cm, 3rd centile) with normal height and weight. The face was long, flat and hypotonic with hypersalivation, an impression of mild hypertelorism, down-slanting palpebral fissures, mild ptosis, flat nasal bridge, anteverted nares, long flat philtrum and mild micrognathia. He had large ears, low posterior hair line, mild pectus excavatum, mildly wide spaced mammillae, bilateral 5th finger camptodactyly, mild cutaneous syndactyly of toes 2 and 3, and a 1×1.5 cm depigmented spot on the leg. The facial features of this patient resembled patient 1 with a *SATB2* deletion published by Rosenfeld *et al*.[26] Psychomotor development was mildly delayed with walking age 18 months and fine motor problems, but expressive speech development was remarkably delayed with few single words, hyperactivity and stereotypic movements at the age of 4 years.

Although a doubled row of upper incisors has not been previously described in *SATB2* defects, some patients had tooth abnormalities such as missing teeth, abnormally shaped and crowded teeth, malocclusion and diastema. Moreover, in E17.5 Satb2−/− mouse embryos the incisor teeth, which express high levels of SATB2 in the wild type, were missing, while the molars, which do not express SATB2 in the wild type, were unaffected.[23]

Table 2 Summary of the patients with deletions affecting *CTNND2*

	Girirajan *et al.* 2013 (patient 12289.p1)	Patient 1 (decipher 284528)	Patient 2 (decipher 248402)	Patient 3 (decipher 269928)	Patient 4 (decipher 271234)
Gender	N/A	Female	Female	Male	Male
deletion affecting *CTNND2*	93 kb deletion, exons 4–9 in-frame (hg18, chr5: 11398907-11491980)	113 kb deletion, exons 4–7 out-of-frame (hg18, chr5: 11431816-11545236)	413 kb deletion, exons 2–8 out-of-frame (hg19, chr5: 11349694-11763030)	479 kb deletion, exons 1–3+5′UTR out-of-frame/ haploinsufficiency (hg19, chr5: 11505316-11985200)	154 kb deletion, exon 3 out-of-frame (hg19, chr5: 11432332-11587173)
Inheritance	De novo	De novo (parents had no specific features and had higher education)	Paternal (father had problems with concentration. He finished lower vocational technical school)	Maternal (mother had no specific features)	Maternal (mother had low normal intelligence)
Additional rare CNVs of coding region	N/A	–	–	452 kb paternally inherited duplication encompassing *WSB1* (hg19, chr17: 25403446-25854990)	–
Weeks of gestation	N/A	Term	40 (C-section for breech position)	37.5	42
Birth measurements	N/A	BW: 3660 g (50th–90th centile), BL: 50 cm (50th–90th centile) and OFC: 35 cm (50th–90th centile)	BW: 4290 g (>97th centile), BL: 54 cm (75th–90th centile)	BW: 3700 g (75th–90th centile), BL: 51 cm (50th–75th centile), OFC : 36 cm (75th–90th centile)	BW: 4280 g (75th–90th centile) (1.2 SD)
Facial features	N/A	Deep set eyes, prominent cheeks, narrow eyebrows, short inner eye distance (ICD 2.7 cm, 2nd centile), deep, slightly backwards rotated ears, and a bulbous nose with prominent columella. She had mild clinodactyly of the fifth finger, which was present in the healthy brother too.	Open mouth, ptosis, downslanted palpebral fissures, anteverted nares, malar flattening, macrodontia, myopathic facies, short nose, abnormality of globe size	High arched palate	Pigmented nevus on right cheek, mild craniofacial dysmorphism with deep set eyes and prominent cheeks
Developmental milestones	N/A	Walking at 16 months, first words at 8–9 months, 3-word sentences at 18 months	Walking at 24 months, 2-word sentences at 36 months	Normal initial motor development, but fine motor problems, difficulties with social skills, delayed language	Unaided sitting at 12 months, walking at 36 months
IQ	N/A	WISC-IV at age 8-year IQ 77, with better language than nonverbal functions, the cognitive profile remained stable at follow-up	SON-R at age 4- year IQ 72	The last WISC IV evaluation results were VCI=74, PRI=67, PSI=86, WMI=67	Attends special school for children with both physical and intellectual disability. At 15 years has receptive and expressive language skills equivalent to 5 years old
Neurologic manifestations	Autism	Short attention span, poor executive functioning and impaired working memory, and immature social-emotional behaviour	Autism, mild intellectual disability, muscular hypotonia, nasal speech	Learning difficulties behavioural problems, diagnosis of autism spectrum disorder	Ataxic cerebral palsy was diagnosed at age 3 years. At 15 years, he had marked axial hypotonia with hyperreflexia, rigidity on passive movements of distal joints and difficulty in initiating movement. Recent unexplained loss of motor skills
MRI	N/A	N/A	N/A	N/A	New changes in internal capsule and basal ganglia at the age of 15 years
Latest measurements	N/A	At 10 years 4 months, her weight, height, and head circumference were 39.6 kg (93rd centile), 145.9 cm (96th centile) and 52 cm (46th centile), respectively. Father's height was 189 cm and mother's 169 cm.	At 11 years 5 months, her weight, height, and head circumference were 60 kg (+1.9 SDS), 167.6 cm (+2.3 SDS) and 55 cm (+0.9 SDS), respectively. Father's height was 180 cm.	He was tall (+3 SD) with OFC=56 cm (+2.5 DS). Father's height was 189 cm. Mother's height was 160 cm.	Unable to obtain height as wheelchair bound. OFC at age of 16.4 years was 52.9 cm (−2.2 SD).
Other features	N/A	–	Hyperextensibility of the finger joints, slender finger, joint laxity, narrow foot, mild scoliosis, accelerated skeletal maturation, long phalanx of finger	Genu valgum	Delayed puberty

BW, birth weight; BL, birth length; CNV, copy number variant; ICD, inner canthal distance; OFC, occipitofrontal circumference; WISC-IV, Wechsler Intelligence Scale for Children-IV.

DNM3 intragenic 372 kb deletion of uncertain significance in patient 59248 with epileptic encephalopathy

DNM3 (MIM *611445) functions in endocytosis of presynaptic vesicles after release of neurotransmitter and postsynaptic receptors,[29] and there is evidence for its interaction with mGluR5 and Homer and its role in dendritic spine morphogenesis.[30] *DNM3* was considered the critical gene for the neurodevelopmental features in patients with larger deletions of 1q24q25, which in addition to severe cognitive disability show a recognisable phenotype including prenatal-onset microcephaly, growth deficiency, small hands and feet with distinctive brachydactyly and distinctive facial features.[31]

CMA showed a 372 kb de novo deletion within the gene *DNM3*, encompassing exons 2–15 in our patient. This girl was referred for severe hypotonia since birth, profound developmental delay, refractory seizures, oedematous hands and feet with tapering fingers and facial dysmorphism. Because of severe epileptic encephalopathy and oedema but absence of typical features such as cerebellar and optic atrophy, a clinical diagnosis of progressive encephalopathy with edema, hypsarrhythmia and optic atrophy (PEHO)-like syndrome was proposed. However, CNV analysis and Sanger sequencing of *DNM3* in eight similar patients with PEHO or PEHO-like syndrome did not reveal any pathogenic finding. This deletion remains of uncertain significance because seizures are only reported in a minority of patients with larger 1q24-q25 deletions and because of the mild phenotype consisting of attention deficit hyperactivity disorder (ADHD) and autism in DECIPHER patient 288412 with a 400 kb deletion limited to *DNM3*.

WES of our patient and both healthy parents revealed a heterozygous de novo missense mutation in *ADAM7* (c.190A>G; p.K64E; chromosome 8g.24304732A>G). Since no germline mutation has been reported so far for *ADAM7*, the relevance of this finding remains also unclear.

Likely benign 19 kb de novo deletion affecting *TH1L* (*NELFCD*) and *CTSZ* in patient 45333

Both genes (MIM *605297 and *603169) are widely expressed in fetal and adult tissues[32] and TH1L interacts with A-Raf kinase, an important intermediate of the growth factor Ras-MAP kinase pathway.[33] CMA revealed a 19 kb de novo deletion partially affecting *TH1L* and *CTSZ*. This boy had macrocephaly, severe ID, hypotonia, haemangioma of the upper lip, bilateral postaxial foot polydactyly and obesity. There was no strong evidence for the pathogenicity of this aberration, and larger overlapping deletions reported in the DECIPHER database (eg, numbers 250209 and 250318) had a divergent phenotype of short stature and microcephaly. Eventually WES revealed the recently reported *PIK3CA*, c.2740G>A, p.G914R mutation in mosaic form causing the megalencephaly capillary malformation syndrome,[34] which fully explains the patient's phenotype. The mutation was confirmed by Sanger sequencing in blood (~7%) and saliva (~16%) and occurred de novo.

Likely benign 122 kb de novo duplication affecting *TAOK3* and *PEBP1* in patient 62848

TAOK3 encodes the serine/threonine-protein kinase TAO3 that acts as a regulator of the p38/MAPK14 stress-activated MAPK cascade involved in the G2/M transition DNA damage checkpoint.[35] PEBP protein is an inhibitor of the Raf/MEK/MAP kinase signalling cascade and functions in the regulation of cell cycle.[36] Therefore, both genes have critical roles in the regulation of cell cycle and can be dosage sensitive. CMA showed a de novo 122 kb duplication within *TAOK3* and *PEBP1* (MIM *604591) in a girl with microcephaly, mild developmental delay and hyperactivity. So far, no polymorphic variants overlapping with this duplication have been reported and a smaller duplication limited to *TAOK3* was reported in DECIPHER (#250362) in a patient with microcephaly and developmental delay, but was reported as inherited from a healthy parent. Eventually WES revealed the *SHANK2* (ENST00000338508) de novo c.2669_2670insC mutation, which causes a frameshift introducing a premature stop codon (p.P891Sfs*32), and explains the patient's phenotype. However, we cannot exclude a multiple hit aetiology in this patient.

Homozygous CNVs

We found two rare homozygous deletions with heterozygous healthy parents (see online supplementary table S2). One, an 83 kb homozygous deletion on 2p21 in two siblings with hypotonia-cystinuria syndrome without cystinuria, further refined the genotype–phenotype correlation in this known ID region and was described and discussed in detail elsewhere.[37]

The second was a 7 kb homozygous frameshift deletion encompassing the first exon of *ACOT7* (isoform ENST00000377855) in a patient with ID, epilepsy and abnormal behaviour. So far no human disorder has been described for any of the ACOT proteins. This boy was born at 40+9 weeks of gestation with normal measurements (3780 g, 53 cm). At age 16 months, he developed a generalised mixed myoclonic-tonic absence seizure disorder. Anticonvulsive treatment was discontinued at age 8 years without recurrence of seizures. Since age 15 years, episodes with ravenousness, extreme fatigue and fluctuating alertness were noted and led to cardiologic evaluation without abnormal findings. He had normal body measurements, micrognathia and mild ID with an IQ of 55–65, hyperactivity and abnormal behaviour and spoke in simple sentences but could not take care of himself.

ACOT7 (MIM *602587), formerly known as brain acyl-CoA hydrolase (*BACH*), encodes acyl-CoA thioesterase 7 and is involved in fatty acid metabolism with other ACOTs.[38] *ACOT7* encodes distinct isoforms with tissue-specific expression and subcellular locations and is strongly expressed in human brain cells such as pyramidal cells in the cerebral cortex, as well as in testes and some other tissues.[39 40] Although lowered levels of ACOT7 in patients with suspected mitochondrial fatty acid oxidation disorders have been shown[41] and a derangement of the ACOT7 protein has been detected in the hippocampus of patients with mesial temporal lobe epilepsy,[42] so far, no mutation or CNV within *ACOT7* was linked to any particular disorder. Recent studies of *ACOT7* conditional central nervous knockout mice (KO) showed that ACOT7 counter-regulate fatty acid metabolism in neurons and protects against neural lipotoxicity. Interestingly, the KO mice exhibited behavioural hyperexcitability after fasting when circulating free fatty acids from lipolysis are elevated,[43] which resembles the episodes of ravenousness and fatigue observed in our patient.

In our patient, the homozygous frameshift deletion within the alternatively spliced first exon of isoform ENST00000377855 most likely results in the depletion of its transcription. In other isoforms, however, it is intronic or in the 5'UTR and the possible effect on transcription or splicing remains uncertain. To further elucidate the involvement of this ACOT7 isoform in the patient's phenotype, we investigated its expression in cDNA panels from fetal and adult human tissues and found the highest levels in adult pancreas, testis, brain, lung, prostate and colon (see online supplementary figure S1). Since we found no

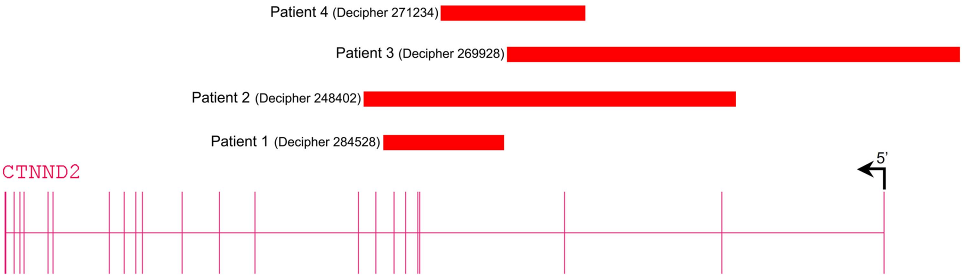
Patient 4 (Decipher 271234)
Patient 3 (Decipher 269928)
Patient 2 (Decipher 248402)
Patient 1 (Decipher 284528)
CTNND2
5'

Figure 2 Schematic representation of *CTNND2* deletions detected in patients. Patient 1 (DECIPHER #284528) with a de novo deletion of exons 4–7, patient 2 (DECIPHER #248402) with a paternally inherited deletion encompassing exons 2–8, patient 3 (DECIPHER #269928) with a maternally inherited deletion of exons 1–3 and 5′UTR and patient 4 (DECIPHER #271234) with a maternally inherited deletion of exon 3.

expression in control lymphoblasts, we were not able to perform expression studies in the patient. Given the similarity to the KO mice phenotype and the segregation of the deletion in the family with heterozygosity in both healthy parents and the healthy brother and absence of the deletion in the healthy sister, it is likely that the homozygous deletion is pathogenic. Since the same deletion has been observed in the heterozygous state in 1 out of 1038 worldwide Affymetrix controls (~0.1%), in 1 out of 451 controls by Conrad *et al*[5] (~0.2%) and in 13 out of 1151 of 1000 Genomes Consortium controls (~1.1%),[44] the homozygous disease frequency would be ~0.000025–0.003%, which is in line with a very rare disorder (1:33 000–4 000 000).

Inherited heterozygous CNVs

Four inherited CNVs were considered as pathogenic or likely pathogenic affecting genes with reported pathogenic rare de novo/inherited deletions (*AUTS2*, *NRXN3*), deletions of a gene observed only in ADHD patients but not in controls (*GRM8*) or the recurrent microdeletion/duplication region in 16p11.2. These CNVs are described and discussed in the supplementary information. Six of the familial CNVs were found to have some evidence for potential pathogenicity but remained with uncertain significance (VOUS) including one duplication in 20p13 and five deletions affecting *STPG2* (*C4orf37*), *SUCLG2*, *PARK2*, *NDUFV3* and *WDR4*, and *TPK1*, respectively.

Eight CNVs were considered likely benign because of the identification of independent pathogenic mutations fully explaining the phenotype or observation of similar CNVs in new control data (see online supplementary table S2). For the rest of inherited CNVs (22), there was no evidence in favour or against pathogenicity (see online supplementary table S1). Selected genes of 10 familial CNVs suspected for recessive pathogenicity were sequenced for a second hit in the trans allele, which was negative for any pathogenic finding (see online supplementary tables S2 and S3).

Incidental findings related to NDDs

Two of the CNVs found in our cohort, both deletions, were classified as incidental findings related to NDDs. The first CNV was a 203 kb deletion within the *DMD* gene (hg18, chrX: 31598556-31801270) (in-frame, exons 49–53 (isoform ENST00000357033), c.7099-?_7872+?del) in a 4-year-old male patient with developmental delay, ptosis and some other facial features, but no sign of muscular dystrophy and normal muscle enzymes. Despite the recent report of X-linked ID in a family with a 3 bp DMD deletion affecting the Dp71 isoform without muscular dystrophy,[45] the deletion in our patient does not affect this isoform and is less likely to explain his phenotype. Therefore, it was considered as an incidental finding with prognostic value for the patient.

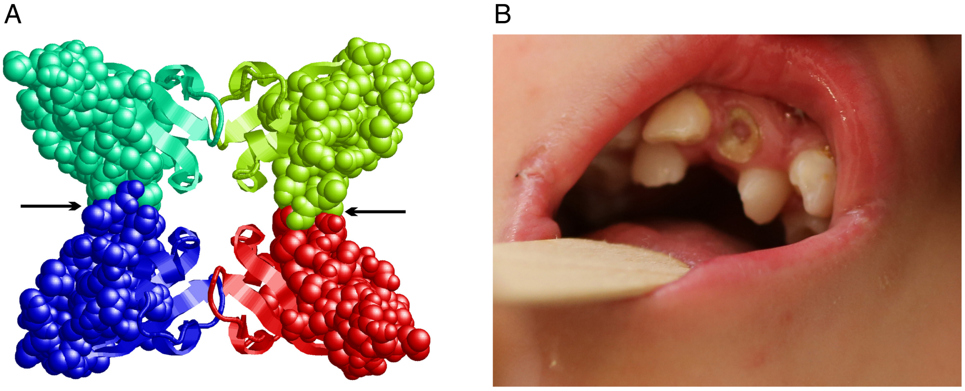

Figure 3 Structure of the Satb2 tetramerisation domain and upper incisors in the patient with intragenic *SATB2* duplication. (A) The four subunits of the tetramer are shown in different colours and those parts, which are duplicated in the mutant, are shown in space-filled presentation. This duplication will affect the interfaces between the dimers that form the tetramer (black arrows). Thus, the intragenic duplication is expected to hamper formation of the tetramer, which was suggested to play an important role for long-range chromatin organisation and coordination in gene regulation.[12] (B) Double row of upper incisors in the patient with 32 kb pathogenic duplication within *SATB2* at age 3 3/12 years.

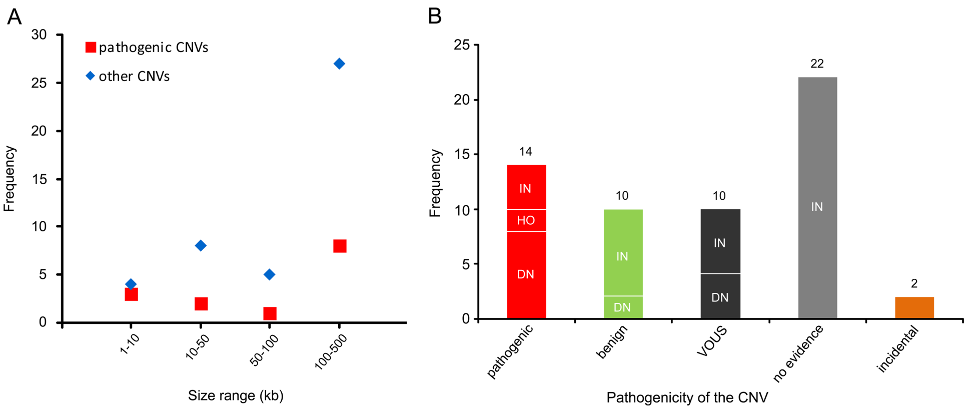

Figure 4 Distribution of copy number variants (CNVs) <500 kb in different size ranges and categories. (A) Frequency of pathogenic or likely pathogenic CNVs (pathogenic) versus other CNVs in four size ranges is shown. (B) Frequency of CNV inheritance pattern in five categories: pathogenic or likely pathogenic (pathogenic), likely benign (benign), variants of uncertain significance (VOUS), CNVs with no evidence in favour or against their pathogenicity (no evidence), and incidental findings related to NDDs (incidental). De novo or likely de novo CNVs are indicated as DN, inherited or likely inherited as IN, and homozygous as HO.

The second CNV was a 380 kb maternally inherited deletion encompassing 10 genes (hg18, chrX: 70006030-70385683) in a female patient later diagnosed with a truncating mutation in *ASXL1* confirming the clinical diagnosis of Opitz-Bohring syndrome.[46] However, the deletion contained several known X-linked recessive disease genes and the patient, carrier mother and grandmother showed 98% skewing of X-inactivation. Therefore, it was considered as an incidental finding with predictive value of pathogenicity in males for future pregnancies.

DISCUSSION

Recent genome-wide studies have shown a significant increase in the burden of rare exonic CNVs in patients with autism spectrum disorder (ASD) compared with controls[7 8] and global burden of rare genic deletions of <500 kb compared with all CNVs,[47] but did not investigate the clinical significance of individual rare CNVs. In this study, we investigated all rare exonic CNVs sizing 1–500 kb detected by genome-wide high-resolution CMA for genetic diagnosis and gene discovery in a large cohort of 714 clinically well-characterised patients with NDDs. 60.4% of such CNVs were confirmed by secondary testing and as expected, false positive aberrations were significantly smaller (median 19 vs 131 kb) in size. However, three out of seven true CNVs sizing 1–10 kb, but only 8 out of 35 sizing 100–500 kb were pathogenic, indicating the highest fraction of pathogenic CNVs in the smallest size range (figure 4A). Therefore, genome-wide exon-level CNV testing would be desirable and may be achievable by next-generation sequencing in the near future.

Although both intragenic deletions and duplications can lead to out-of-frame defects and gene haploinsufficiency, frequency of intragenic deletions appears to be higher than duplications. A study with targeted exon-level CNV analysis in 3018 patients with suspected Mendelian disorders illustrated a CNV detection rate of 3.3% of which 96 were deletions and only 2 were duplications.[48] Recent data from WES have also indicated the enrichment of 1–30 kb deletions in individuals with ASD.[7] Accordingly, 12 of our 41 confirmed rare small deletions (29%) were pathogenic, but only 2 of 17 confirmed rare duplications (12%) were categorised as such, but the difference did not reach statistical significance.

With reference to inheritance pattern (figure 4B), 24% of confirmed small CNVs were de novo or likely de novo, but only 57% of these were considered disease causing. Rare CNVs occurring de novo are more likely to be pathogenic, and consensus guidelines suggest de novo CNVs to be considered for causality of the abnormal phenotype.[4] However, there are few reported instances in which candidate pathogenic de novo CNVs <500 kb eventually appeared to be benign, indicating that their causality should not be overestimated.[49] Likewise we found 2 of 14 (14%) de novo rare CNVs <500 kb to be likely benign because of the identification of pathogenic mutations in known disease genes by WES in the corresponding patients, fully explaining their phenotypes (table 1; patients 62848 and 45333). For the two pathogenic de novo CNVs affecting novel disease loci, overlapping CNVs in patients with similar phenotypes were identified via the DECIPHER database. While we described in detail the novel syndrome associated with *MED13L* haploinsufficiency elsewhere,[13] the phenotype of borderline low IQ with or without autistic features or developmental delay associated with *CTNND2* deletions is first described here.

In our cohort, 71% of confirmed small CNVs were heterozygous and familial. Recent studies indicated enrichment of inherited CNVs in patients with mild clinical phenotype[50] and sporadic ASD cases,[8] as well as lower cognitive performance in controls carrying rare CNVs.[51] However, clinical interpretation of such CNVs in individual cases remains challenging. We found four inherited heterozygous deletions (10% of confirmed familial heterozygous CNVs) to be pathogenic or likely pathogenic based on the parent's phenotype and/or reported cases in the literature. Notably, eight (20%) of the inherited small CNVs could be categorised as likely benign after finding clearly pathogenic point mutations in the patients or observing similar CNVs in new control data (see online supplementary table S2) and the rest remained without clear evidence (see online supplementary table S1; figure 4B).

In addition, our findings underline the importance of homozygous disease causing CNVs <500 kb (~0.3% of total cohort) in a genome-wide evaluation. Pathogenic homozygous CNVs are more commonly described in consanguineous families, but limited data are available on the genome-wide estimate of these CNVs with a reported frequency of ~0.009% in a cohort of diverse clinical phenotype[52] and ~0.5% in 194 patients with ASD.[53] Here, both of our homozygous deletions were detected in patients of non-consanguineous parents of Swiss origin, the homozygous 2p21 deletion at the border of a 7.6 Mb loss of heterozygosity (LOH) region[37] and the *ACOT7* exonic homozygous deletion flanked by two heterozygous single-nucleotide polymorphism within a 1.7 Mb interval. CNVs or mutations affecting *ACOT7* have not been reported before; however, given the familial segregation and the overlap with the reported KO mice phenotype, we suggest that *ACOT7* indeed causes a novel autosomal-recessive disorder characterised by mild ID, epilepsy and episodes of ravenousness and fatigue.

In our cohort, the diagnostic yield of larger pathogenic CNVs sizing 500 kb–10 Mb was 8.8%, which is slightly lower than reported data and may be explained by the clinical preinvestigation of all patients, which allowed 13 cases with recognisable microdeletion syndromes to be diagnosed by targeted testing (deletion 22q11.2, Williams–Beuren syndrome, Smith–Magenis syndrome). These diagnoses, which are frequently reported in CMA studies, would have added another 1.8% to this cohort seen in our genetic clinic and we also excluded patients with potentially cytogenetically visible CNVs larger than 10 Mb. The overall added value of rare CNVs <500 kb to the diagnostic yield was ~2% (1.1% de novo, 0.3% homozygous, 0.6% inherited). Of note, 79% of this diagnostic yield represented CNVs overlapping with known disease loci while 21% affected novel loci and were contributory to delineation of novel disease entities in the course of this study. In addition, two of the confirmed small CNVs (0.3% of patients) represented incidental pathological findings not related to the patient's current phenotypes. Our finding of 11 out of 714 patients (1.54%) with small pathogenic CNVs in known disease loci is higher than the 0.4–1% observed in previous studies investigating more than 300 patients each using lower resolution Agilent 44k, 105k, 180k, 244k arrays or custom-designed exon-targeted arrays.[54–57]

In summary, including the 0.4% of patients with pathogenic CNVs in novel disease loci, our results verify the diagnostic relevance (~2%) of genome-wide rare CNVs <500 kb and their inherent potential to discover new conditions enabling better characterisation of NDDs.

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Supplementary Data

The clinical significance of small copy number variants in neuro-developmental disorders

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Supplementary Table 1. List of all CNVs <500 kb detected in 714 patients with Affymetrix high resolution arrays

Patient ID	Gender	CNV	Chr	Band	hg, chr:start-end	Size (kbp)	Confidence value (%)	Marker count	Affected gene(s)	MLPA target (s)	MLPA result	Inheritance	Pathogenicity	Affymetrix array	Remarks
59484	M	Del	4	q12	hg18, 4:52554386-52556739	2	96	7	LRRC66	LRRC66 e4	Confirmed	Paternal	no evidene	2.7	
61587	M	Del	2	q31.1	hg18, 2:170957153-170959604	2	96	6	MYO3B	MYO3B e15, e16	Confirmed	Paternal	no evidene	2.7	
60984	F	Del	16	p12.1	hg18, 16:24793142-24797112	4	96	6	SLC5A11	SLC5A11 e6, e7	Confirmed	Maternal	no evidene	2.7	Sanger sequencing of the gene was negative. SLC5A11 is a cotransporter. Cotransporters represent a major class of proteins that make use of ion gradients to drive active transport for the cellular accumulation of nutrients, neurotransmitters, osmolytes, and ions. Many members of this protein family are involved in genetic disorders.
617	F	Del	17	p13.3	hg18, 17:2519114-2523280	4	96	6	PAFAH1B1	PAFAH1B1 e6, e7	Confirmed	Parents N/A	Pathogenic	2.7	PAFAH1B1 is OMIM gene for incomplete lissencephaly.
9229	M	Del (homo)	1	p36.31	hg18, 1:6361008-6368114	7	98	8	ACOT7	ACOT7 e1, i1-2	Confirmed	Parental (het)	Likely pathogenic	2.7	He is the only child in the family with homozygous deletion and is the only affected. Details are available in the descripton of the patient.
58822	M	Del	7	q11.22	hg18, 7:69233202-69240841	8	93	16	AUTS2	AUTS2 i3-4, e4	Confirmed	Paternal	Pathogenic	2.7	Father is affected. Sanger sequencing of the gene was negative. Microdeletions of the gene in patients with variable neurodevelopmental features have been reported.
68629	M	Del	11	q13.4	hg19, 11:74569938-74579385	9	92	19	XRRA1	XRRA1 i11-12, e12	Confirmed	Maternal	no evidene	Cytoscan	
61825	F	Del	19	q13.11	hg18, 19:39379364-39389949	11	92	14	LSM14A	LSM14A e3, i3-4	Confirmed	Paternal	no evidene	2.7	
62610	M	Del	2	p16.2	hg18, 2:54411976-54424374	12	91	9	C2orf73	C2orf73 e2, e3	Confirmed	Paternal	no evidene	2.7	Only exon 2 of C2orf73 was deleted.

62611	F	Del	4	q22.1	hg18, 4:88438703-88452522	13	90	14	HSD17B13	HSD17B13 i6-7, e7	Confirmed	Paternal	Likely benign	2.7	Heterozygous de novo mutation in SCN2A: c.4025T>C (p.L1342P) was found in this patient. There are DGV variations 30208 (2/485 ctls, Jakobsson 2008), 51479 (3/2026 ctls, Shaikh 2009) and 36311 (1/1 ctl, Kidd 2008) with deletions encompassing some other exon(s) of HSD17B13.
56366	F	Del	12	q24.21	hg18, 12:115158648-115175505	17	87	18	MED13L	MED13L i1-2, e2	Confirmed	*de novo*	Pathogenic	2.7	Sanger sequencing of the gene was negative. Whole-exome sequenicng showed no evidence for a non-allelic second hit. This patient and further cases were described elsewhere (EJHG 2013).
45333	M	Del	20	q13.32	hg19, 20:57556968-57575495	19	94	28	TH1L (NELFCD), CTSZ	TH1L (NELFCD) e2, e10; CTSZ e5	Confirmed	*de novo*	Likely benign	Cytoscan	Pathogenic mosaic heterozygous mutation in PIK3CA: c.2740G>A (p.G914R) was found in the patient by whole-exome sequencing.
68738	M	Del	7	q35	hg19, 7:144509480-144534911	25	93	35	TPK1	TPK1 i1-2, i2-3	Confirmed	Father N/A	VOUS	Cytoscan	This deletion was absent in the mother and a healthy sister but present in the likewise affected sister. Father's DNA was not available. Sanger sequencing of the gene in available samples was negative. TPK1 encodes thiamin pyrophosphokinase 1 which catalyzes the conversion of thiamine to thiamine pyrophosphate. It is expressed in brain, and a de novo missense mutation of TPK1 has been reported in an autism cohort using WES by Sanders et al.
31553	M	Del	21	q22.3	hg18, 21:43169759-43201708	32	92	22	NDUFV3, WDR4	NDUFV3 e3	Confirmed	Paternal	VOUS	2.7	Sanger sequencing of the gene was negative. A de novo missense mutation of WDR4 has been reported in an autism cohort using WES and mutations of NDUFV3 have been reported in complex I deficiency. WDR4 is a candidate for some disorders mapped to 21q22.3 as well as for the development of Down syndrome phenotypes. NDUFV3 encodes NADH dehydrogenase (ubiquinone) flavoprotein 3 which is one of the subunits that make up the NADH-ubiquinone oxidoreductase complex.

70886	M	Dup	2	q33.1	hg19, 2:200278502-200310272	32	89	68	SATB2	SATB2 i3-4, e4	Confirmed	*de novo*	Pathogenic	Cytoscan	Deletions and truncating mutations of SATB2 have been implicated as causative for cleft palate and ID.
64035	M	Del	8	q24.3	hg18, 8:141650841-141693661	43	92	43	EIF2C2 (AGO2)	EIF2C2 (AGO2) e2, e3	Confirmed	Maternal	no evidene	2.7	
60407 & 66928 & 60324	F (all)	Del	4	q12	hg18, 4:52579180-52624947	46	94	52	SGCB, SPATA18	SGCB e2; SPATA18 e4	Confirmed in all	Maternal	no evidene	2.7	They are triplet. Exome sequencing did not reveal any obvious Mendelian cause.
43552	M	Del	16	p13.3	hg18, 16:4986264-5046682	60	89	53	NAGPA, C16orf89, SEC14L5	NAGPA e4, SEC14L5 e12	Confirmed	*de novo*	VOUS	2.7	A smaller deletion including only SEC14L5 and NAGPA is present in 1/1038 of a world wide control cohort by Affymetrix.
63660	M	Del	12	q24.31	hg18, 12:120240193-120307177	67	88	45	ANAPC5	ANAPC5 e4, e10	Confirmed	Maternal	no evidene	2.7	
70229	M	Del	12	q24.33	hg19, 12:132552537-132623611	71	90	48	EP400, EP400NL, DDX51	EP400 e51, EP400NL e3	Confirmed	*de novo*	VOUS	Cytoscan	There is patient 262376 in Decipher database with a duplication of unknown inheritance encompassing the same genes.
62915	F	Del	X	p22.2	hg18, X:16531546-16605488	73	91	57	S100G, CTPS2	CTPS2 e11, e12, e16	Confirmed	Maternal	no evidene	2.7	
50286 & 53032	F & M	Del (homo)	2	p21	hg18, 2:44435674-44519612	83	99	93	PREPL, C2orf34 (CAMKMT)	PREPL e1, C2orf34 e1, e2, e3	Confirmed	Parental (het)	Pathogenic	2.7	Two siblings were affected. It is overlapping hypotonia-cystinuria syndrome. These patients are described elsewhere (AJMG 2013).
54949	M	Del	6	q26	hg18, 6:162072914-162156999	84	92	93	PARK2	N/A	array in parents	Maternal	VOUS	2.7	This deletion was within PARK2. CNVs of PARK2 have been shown by Glessner et al. 2009 and Girirajan et al. 2013 to be significantly enriched in ASD cases versus controls.
72125	F	Dup	10	p14	hg19, 10:7932363-8033508	101	90	108	TAF3	TAF3 e3	Confirmed	*de novo*	VOUS	Cytoscan	TAF3 encodes TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor. TAFs contribute to promoter recognition and selectivity and act as antiapoptotic factors.
62563	F	Del	5	p15.2	hg18, 5:11431816-11545236	113	NA	99	CTNND2	CTNND2 e5, e6	Confirmed	*de novo*	Pathogenic	6.0	Sanger sequencing of the gene was negative. CTNND2 is implicated in the ID phenotype of cri-du-chat syndrome. Further patients are discussed in this paper.
60045	M	Dup	7	q36.1	hg18, 7:148278728-148391821	113	89	60	PDIA4	PDIA4 e7, e8	Confirmed	Paternal	no evidene	2.7	
45679	M	Dup	20	q11.21-q11.22	hg18, 20:31442246-31556613	114	88	72	SNTA1, CDK5RAP1, CBFA2T2	SNTA1 e2, e4	Confirmed	Paternal	no evidene	2.7	

62848	F	Dup	12	q24.23	hg18, 12:117061815-117183853	122	NA	71	TAOK3, PEBP1	TAOK3 e9, e10, e18, e21	Confirmed	*de novo*	Likely benign	6.0	Pathogenic heterozygous mutation in SHANK2: c.2514_2515insG (p.P841Sfs*32) was found in this patient by whole-exome sequencing.
64926	M	Dup	1	p35.1	hg18, 1:33599961-33727852	128	90	100	ZSCAN20, PHC2	ZSCAN20 e2, e3	Confirmed	Maternal	no evidene	2.7	
62709	M	Del	17	q25.3	hg18, 17:73412776-73547163	134	90	90	TNRC6C	TNRC6C e2	Confirmed	Paternal	no evidene	2.7	
63930	M	Del	5	q15	hg18, 5:95753227-95900228	147	92	162	PCSK1	PCSK1 e3, e11	Confirmed	Paternal	no evidene	2.7	This patient has balanced translocation 5/12.
71156	F	Del	16	p13.3	hg19, 16:3788867-3935836	147	91	353	CREBBP	MLPA Kit P245	Confirmed	*de novo*	Pathogenic	Cytoscan	This deletion was within CREBBP which is the OMIM gene for Rubinstein-Taybi syndrome.
56761	M	Del	X&Y	q28	hg18, X:154686877-154843251	156	NA	15	VAMP7	VAMP7 e4, e5	Confirmed	Paternal	Likely benign	6.0	Sanger sequencing of the gene was negative. Patient 272246 in Decipher database with similar inherited deletion, has an additional 10Mb de novo deletion.
68422	M	Dup	1	q43	hg19, 1:236964009-237124627	161	90	236	MTR	N/A	array in parents	Maternal	no evidene	Cytoscan	
55113	M	Del	3	p14.1	hg18, 3:67592633-67762247	170	93	198	SUCLG2	SUCLG2 e5, e9	Confirmed	Maternal	VOUS	2.7	Sanger sequencing of the gene was negative. Patient 265229 in Decipher database with deletion within SUCLG2 (inherited) and patient 259685 with duplication of the whole gene (inherited) have different phenotypes. SUCLG2 encodes a GTP-specific beta subunit of succinyl-CoA synthetase.
62155	M	Del	9	p22.1	hg18, 9:19379757-19555588	176	NA	113	ASAH3L (ACER2), SLC24A2	ASAH3L (ACER2) e2, e4	Confirmed	Maternal	Likely benign	6.0	Sanger sequencing of the gene was negative. Pathogenic hemizygous 4 bp del in RPS6KA3 (RSK2): IVS10+4_+7delAGTA was found in this patient confirming the clinical diagnosis of Coffin-Lowry syndrome.
60245	F	Del	16	q23.3	hg18, 16:81560769-81736858	176	93	259	CDH13	CDH13 e3, e4	Confirmed	Maternal	Likely benign	2.7	Pathogenic heterozygous mutation in PTPN11: c.922A>G (p.N308D) was found in this patient confirming the diagnosis of Noonan syndrome. There are two duplications and five deletions within CDH13 (inherited or undefined) in Decipher database.

68942	M	Dup	X	q25	hg19, X:122803456-122986898	183	94	516	THOC2	THOC2 e1, e3	Confirmed	Maternal	no evidene	Cytoscan	This duplication was also present in maternal grandfather.
60482	M	Dup	1	p33	hg18, 1:46605120-46799318	194	NA	110	DMBX1, KNCN, FAAH, MKNK1	FAAH e3, e11; DMBX1 i1-2, e3	Confirmed	Paternal	no evidene	6.0	
57650	M	Del	X	p21.1	hg18, X:31598556-31801270	203	99	257	DMD	MLPA Kit P034	Confirmed	Parents N/A	Pathogenic, incidental finding	2.7	This deletion within DMD gene, in a male patient with facial dysmorphysim, developmental delay and ptosis but no sign of muscular dystrophy and normal muscle enzymes, was considered as an incidental finding with prognostic value. The deletion does not affect Dp71 isoform.
63153	F	Dup	1	p34.3	hg18, 1:38974598-39179543	205	90	168	GJA9, RRAGC, MYCBP, RHBDL2	GJA9 e2; RRAGC e7	Confirmed	Paternal	Likely benign	2.7	There are three partially overlapping deletions and one duplication in DGV database.
61006	M	Dup	4	q25	hg18, 4:113767097-113982110	215	89	133	LARP7, C4orf21, ANK2	LARP7 e2; C4orf21 e1; ANK2 e1	Confirmed	Maternal	no evidene	2.7	
62789	F	Dup	20	p13	hg18, 20:2635422-2864768	229	NA	136	FAM113A, CPXM1, VPS16, PCED1A, C20orf141, EBF4, PTPRA	VPS16 e22	Confirmed	Father N/A	VOUS	6.0	This dup was absent in the mother but present in the likewise affected sister. Father's DNA was not available. There are patients 254264 and 272274 in Decipher database with similar inherited duplications and ID.
60045	M	Dup	6	p21.1	hg18, 6:42147766-42383555	236	90	212	GUCA1B, GUCA1A, MRPS10, TRERF1, TAF8	GUCA1A e5, e6	Confirmed	Maternal	no evidene	2.7	
60984	F	Dup	X	q22.3	hg18, X:110044420-110289813	245	91	301	PAK3	PAK3 e1, e2	Confirmed	Paternal	Likely benign	2.7	PAK3 is a known X-linked recessive disease gene. In our case, it is a duplication and it is inherited from the healthy father.
59700	M	Dup	19	p13.11	hg18, 19:17283794-17537339	254	NA	81	11 genes	FAM125A (MVB12A) e2; SLC27A1 e2	Confirmed	Maternal	Likely benign	6.0	An overlapping duplication is present in 1/1038 of a world wide control cohort by Affymetrix.
53983	M	Del	7	q31.33	hg18, 7:125890040-126149790	260	93	301	GRM8	GRM8 e7, e8	Confirmed	Paternal	Likely pathogenic	2.7	Sanger sequencing of the gene was negative. Several CNVs of GRM8 have been reported in patients with ADHD compared to controls.
57614	M	Del	3	q12.3	hg18, 3:103671524-103941739	270	NA	141	ZPLD1	ZPLD1 e10, e11	Confirmed	Maternal	no evidene	6.0	

57028	F	Del	4	q22.3	hg18, 4:98745657-99078956	333	93	304	C4orf37 (STPG2)	C4orf37 (STPG2) e10	Confirmed	Paternal	VOUS	2.7	Sanger sequencing of the gene was negative. There are patients 249670 (maternally inherited) with partially overlapping phenotype to our patient and 256952 (inherited from normal parent) with seizure in Decipher database who have intragenic STPG2 deletions.
59248	F	Del	1	q24.3	hg18, 1:170135864-170505998	372	NA	244	DNM3	N/A	confirmed by FISH	*de novo*	VOUS	6.0	Sanger sequencing of the gene was negative. DNM3 encodes dynamin 3, a member of a family of guanosine triphosphate (GTP)-binding proteins that associate with microtubules and are involved in vesicular transport. WES of the patient and healthy parents revealed a heterozygous de novo missense mutation in ADAM7: c.190A>G (p.K64E). Since no germline mutation of ADAM7 has been reported so far, the relevance of this finding remains also unclear.
63409	F	Del	X	q13.1	hg18, X:70006030-70385683	380	93	209	10 genes	IL2RG i4-5, e6; MED12 e9, e13	Confirmed	Maternal	Pathogenic (in males), incidental finding	2.7	This patient was later diagnosed with Opitz-Bohring syndrome with pathogenic mutation in ASXL1:c.2197C>T (p.Gln733X). The deletion contains several known X-linked recessive disease genes and is likely lethal in males. The patient, carrier mother and grandmother showed 98% skewing of X-inactivation.
64717	F	Dup	16	p11.22	hg18, 16:29594946-30038054	443	88	287	25 genes	MLPA Kit P297	Confirmed	Maternal	Likely pathogenic	2.7	Recurrent 16p11.22 microduplications syndrome.
71264	M	Del	17	q21.31	hg19, 17:43703800-44163085	459	90	857	9 genes	MLPA Kit P245	Confirmed	*de novo*	Pathogenic	Cytoscan	Recurrent 17q21.31 microdeletion syndrome.
48459	M	Del	14	q24.3-q31.1	hg18, 14:78321869-78784675	462	92	586	NRXN3	NRXN3 e9, e10	Confirmed	Paternal	Likely pathogenic	2.7	Sanger sequencing of the gene was negative. A mixture of inherited and de novo deletions within NRXN3 has been reported in ASD.
69234	F	Del	X	p11.4	hg19, X:41339667-41811516	472	91	1040	CASK, GPR34, GPR82	CASK e23, e24	Confirmed	*de novo*	Pathogenic	Cytoscan	Phenotypic spectrum associated with CASK loss-of-function has been described.
52253	M	Del	17	q21.31	hg18, 17:41049320-41522088	473	NA	329	9 genes	MLPA Kit P245	Confirmed	Parents N/A	Pathogenic	6	Recurrent 17q21.31 microdeletion syndrome.

57305	M	Dup	21	q21.1	hg18, 21:21541563-22029191	488	NA	331	NCAM2	NCAM2 e10, e12	Confirmed	Paternal	Likely benign	6.0	There is an almost overlapping deletion in Decipher population CNVs (singleton).
62848	F	Del	3	q22.1	hg18, 3:132148497-132640489	492	NA	263	NEK11, ASTE1, NUDT16, NUDT16P, ATP2C1	ATP2C1 e23; NEK11 e16	Confirmed	Paternal	no evidene	6.0	
69551	M	Dup	X	q25	hg19, X:123586540-123589505	3	88	16	ODZ1 (TENM1)	ODZ1 (TENM1) e23	Not Confirmed			Cytoscan	
66314	F	Dup	22	q13.2	hg 19, 22:41530715-41533822	3	94	16	EP300	MLPA Kit P333	Not Confirmed			Cytoscan	
57123	F	Del	5	q12.3	hg18, 5:64893125-64897683	5	93	8	CENPK, PPWD1	CENPK i1-2; PPWD1 e1	Not Confirmed			2.7	It was only detected by annotation 29.
69551	M	Dup	X	p11.4	hg19, X:41392871-41396522	4	91	11	CASK	CASK e23, e24	Not Confirmed			Cytoscan	
56894	F	Del	1	q42.2	hg19, 1:231999534-232003661	4	92	12	DISC1	DISC1 i9-10, e10	Not Confirmed			Cytoscan	
67354	F	Del	9	p24.3	hg19, 9:332170-336058	4	92	12	DOCK8	DOCK8 e11, i11-12	Not Confirmed			Cytoscan	
67674	M	Del	11	p14.2	hg19, 11:26535654-26539603	4	92	12	ANO3	ANO3 i5-6, i6-7	Not Confirmed			Cytoscan	
65010	M	Del	5	q12.1	hg18, 5:61926273-61935437	9	93	12	IPO11	IPO11 i27-28, e28	Not Confirmed			2.7	It was only detected by annotation 29.
63935	M	Del	X	q28	hg18, X:147733407-147741983	9	95	10	AFF2	AFF2 e6, e7, i7-8	Not Confirmed			2.7	
60984	F	Del	4	p14	hg18, 4:39021615-39032347	11	89	12	RFC1	RFC1 e2, e3	Not Confirmed			2.7	It was only detected by annotation 29.
66075	F	Del	11	q14.1	hg18, 11:77960543-77971422	11	88	14	NARS2	NARS2 e1, e2	Not Confirmed			2.7	
64622	M	Del	11	q21	hg18, 11:93862415-93873098	11	88	15	MRE11A, ANKRD49	MRE11A e1, ANKRD49 i2-3	Not Confirmed			2.7	
38048	M	Del	10	q22.1	hg18, 10:71953731-71965051	11	90	12	KIAA1274 (PALD1)	KIAA1274 (PALD1) e2, e3, e5, e6	Not Confirmed			2.7	This patient was later diagnosed with acrocallosal syndrome with pathogenic compound heterozygous mutation in KIF7.
38004	F	Del	1	p31.3	hg18, 1:66862092-66873951	12	90	15	SGIP1	SGIP1 e2, e3, e4	Not Confirmed			2.7	

67116	F	Del	5	q33.1	hg18, 5:151746458-151759934	13	86	12	NMUR2	NMUR2 e2, e3	Not Confirmed	2.7	
66075	F	Del	4	q25	hg18, 4:113765627-113779348	14	88	17	LARP7, C4orf21	LARP7 e1; C4orf21 e1	Not Confirmed	2.7	It was only detected by annotation 29.
64103	M	Del	14	q21.31	hg18, 14:41498859-41512482	14	87	19	KIAA1267 (KANSL1)	KIAA1267 (KANSL1) e4, e5	Not Confirmed	2.7	
68198	M	Del	1	p35.1	hg18, 1:32918077-32933954	16	89	17	SYNC, RBBP4	SYNC e1, e2, e3	Not Confirmed	2.7	
62783	M	Del	2	q36.1	hg18, 2:223459673-223479127	19	88	19	ACSL3	ACSL3 i2-3, e3	Not Confirmed	2.7	
65880	M	Dup	X	q25	hg19, X:123541092-123559735	19	85	44	ODZ1 (TENM1)	ODZ1 (TENM1) e23, e24	Not Confirmed	Cytoscan	
60439	M	Del	15	q26.3	hg18, 15:97948427-97967301	19	88	21	MEF2A	MEF2A i1-2, i2-3	Not Confirmed	2.7	
67687	F	Del	9	q21.11	hg18, 9:72418692-72446917	28	88	30	TRPM3	TRPM3 e11, e12	Not Confirmed	2.7	
61186	M	Del	1	p36.21	hg18, 1:15829278-15860394	31	86	33	DDI2, RSC1A1	DDI2 e6, e9	Not Confirmed	2.7	
62075	F	Del	7	p12.2	hg18, 7:49959248-49994235	35	87	42	ZPBP	ZPBP e7, i7-8	Not Confirmed	2.7	
56985	M	Dup	6	q16.3	hg18, 6:102585885-102621667	36	89	48	GRIK2	GRIK2 e14, e15	Not Confirmed	2.7	It was only detected by annotation 29.
58404	F	Del	1	p34.3	hg18, 1:36197451-36234202	37	86	40	EIF2C3 (AGO3)	EIF2C3 (AGO3) i3-4, e5, i5-6	Not Confirmed	2.7	
66075	F	Del	3	q29	hg18, 3:197974381-198016767	42	85	51	PAK2	PAK2 e2, e3	Not Confirmed	2.7	
60407 & 66928 & 60324	F (all three)	Del	18	q21.1	hg18, 18:43620216-43668188	48	88	31	SMAD2	SMAD2 e3, e6	Not Confirmed	2.7	They are triplet.
62924	M	Dup	11	q13.5	hg18, 11:76468150-76561055	93	88	63	OMP, MYO7A, CAPN5	MYO7A e14, e15; CAPN5 e5, e9	Not Confirmed	2.7	
48307	M	Dup	4	p16.1	hg18, 4:8994976-9095173	100	NA	52	DEFB131, LOC650293	DEFB131 e1	Not Confirmed	6.0	

48307	M	Del	5	p15.33	hg18, 5:723744-826556	102	NA	34	TPPP	TPPP e2	Not Confirmed	6.0
60106	M	Dup	4	q13.3	hg18, 4:72549816-72662940	113	90	142	SLC4A4	SLC4A4 e19, e25	Not Confirmed	2.7
61186	M	Dup	18	q22.3	hg18, 18:70252255-70369954	118	88	73	C18orf51 (FAM69C), CNDP2, CNDP1	C18orf51 (FAM69C) e3, CNDP2 e6	Not Confirmed	2.7
62783	M	Dup	8	q24.3	hg18, 8:141587386-141711847	124	88	112	EIF2C2 (AGO2), CHRAC1	EIF2C2 (AGO2) e2, e3	Not Confirmed	2.7
64170	F	Dup	X	p11.22	hg18, X:50147702-50277014	129	88	131	DGKK	DGKK e1, e4	Not Confirmed	2.7
66075	F	Dup	X	p22.11	hg18, X:23210130-23347055	137	88	132	PTCHD1	PTCHD1 e2, e3	Not Confirmed	2.7
62075	F	Dup	X	q22.1	hg18, X:101379750-101531945	152	88	131	NXF2, NXF2B	NXF2 e3, e23	Not Confirmed	2.7
61098	M	Dup	19	p13.3	hg18, 19:2096867-2277988	181	NA	44	10 genes	DOT1L e5, e6	Not Confirmed	6.0

Pathogenic or likely pathogenic inherited heterozygous CNVs

patient 58822, 8 kb deletion within *AUTS2*, paternally inherited (pathogenic)

AUTS2 (autism susceptibility candidate 2, MIM *607270) was first discovered as a candidate gene when it was found to be disrupted by a balanced translocation in a pair of monozygotic twins with autism, developmental delay and epilepsy.[1] Despite some functional studies indicating its nuclear location, brain expression in various cell types as well as in regions implicated in autism spectrum disorder (ASD) and its potential role in neuronal development,[2-3] exact function of the gene and its pathways are unknown.

We found an 8 kb in-frame deletion encompassing exon 4 of *AUTS2* in a male patient with mild developmental delay, growth deficiency, microcephaly, cryptorchidism, bilateral inguinal and umbilical hernia, and minor anomalies including clinodactyly of the 5th finger, mild interdigital webbing and joint laxity. At the age 5 years 10 months height was 103 cm (<3rd centile), weight 16 kg (3rd centile), OFC 49 cm (<3rd centile) and eruption of teeth was delayed with first tooth at the age of 1 year. He was speaking in full sentences but was shy and had some mild motor delay with balancing problems and reduced pain sensitivity. This deletion was paternally inherited and the father was likewise affected with relative short stature (168.2 cm (10th-25th centile), OFC 55cm (10th centile)) in comparison to his parents, brother and sisters who had a height of about 180 cm.

Various aberrations disrupting *AUTS2* have been linked to ID, developmental delay and ASD. In addition, many of the reported patients have had additional manifestations such as epilepsy, microcephaly, facial dysmorphisms and short stature.[4-6] A recent study reported 24 exonic microdeletions of the gene in patients with variable neuro-developmental features, growth and feeding problems, skeletal abnormalities, and congenital malformations introducing an AUTS2 syndrome. They indicated that dysmorphic features and ID were more pronounced in individuals with 3' deletions because these affect also the alternative transcript of exons 9-19, which is expressed in brain.[4] The finding of the mild phenotype in our family with exon 4 deletion, which is 5' of the alternative transcription start, further supports this genotype-phenotype correlation and emphasises mild short stature as a major clinical finding.

Possible regulatory function of AUTS2 is in line with the presence of several protein interaction motifs for SH2, SH3, and WW domains, as well as the existence of numerous phosphorylation sites.[3] Since no functional domains were reported for exon 4, protein interaction motifs and phosphorylation sites were predicted using the eukaryotic linear motif (ELM) databank.[7] Phosphorylation sites were additionally verified using the NetPhosK server.[8] The absence of exon 4 resulting in the deletion of amino acids 209-220 affects a sequence stretch that contains an unusually high number of residues that are predicted to become phosphorylated: S207, S209, S210, S213, S214, T217, and Y219. Interestingly, the spacing of the serine residues meets exactly the preferences for the action of the hierarchical protein kinases CK1 and GSK3.[9] Residues T217 and Y129 are part of the typical TGY dual-phosphorylation motif of MAP-kinase p38, which becomes phosphorylated by kinases of the MKK family during cell cycle regulation.[10]

patient 64717, 443 kb duplication in 16p11.2, maternally inherited (likely pathogenic)

Recurrent reciprocal 16p11.2 CNVs are characterised by a spectrum of neuro-cognitive and psychiatric phenotypes that are subject to incomplete penetrance and variable expressivity.[11-12]

CMA revealed a 443 kb maternally inherited duplication on 16p11.2 (MIM #614671) encompassing 25 genes in the girl referred for global developmental delay, muscular hypotonia, mild unsteady gate, and overgrowth. She was born spontaneously at term with weight 4500 g (>97th centile) and length 50 cm (25th centile). Developmental milestones were delayed (walking age 2 years, first words at ~4 years). At the age of 6 years and 8 months, her weight, height and head circumference were 31.9 kg (>97th centile), 131 cm (>97th centile; father: 180 cm, mother: 170 cm) and 50 cm (10th-25th centile). Physical examination revealed brachycephaly, relatively short neck, round face with prominent cheeks, narrow eyebrows, hypertelorism, broad philtrum, high-arched palate and large ears (6 cm; 98th centile). Since recurrent 16p11.2 CNVs have been associated with developmental delay, ID, autism, ADHD, seizures, and psychiatric problems and the spectrum of abnormal phenotypes has been expanded to include congenital abnormalities,[12] it is very likely that the duplication is pathogenic in this patient with incomplete penetrance or variable expressivity since the mother's cognition was not formally tested.

patient 48459, 462 kb deletion within *NRXN3*, paternally inherited (likely pathogenic)

NRXN3 (MIM *600567) encodes neurexin 3 in alpha- and beta isoforms expressed at variable levels throughout the brain. The neurexins are highly expressed in presynaptic terminals and have been shown to have important roles in synaptic cell adhesion and neurotransmitter secretion.[13]

CMA revealed a 462 kb paternally inherited deletion encompassing exons 6-12 of the alpha isoform of *NRXN3* and reaching very closely to the transcription start of the beta isoform

(Figure S2). Sanger sequencing of the gene in the patient revealed no pathogenic mutation. This boy was referred due to mild learning difficulties, minor motor problems, dilated cardiomyopathy, some facial features and anal atresia. Deletions of *NRXN3* have been recently reported in patients with ASD (one de novo, one inherited from the father with borderline autism phenotype, and two inherited from apparently healthy parents) with reduced penetrance and variable expressivity.[14] Therefore, the *NRXN3* deletion likely contributes to the learning difficulty in our patient, but the cause of his physical problems remains unknown.

patient 53983, 260 kb deletion within *GRM8*, paternally inherited (likely pathogenic)

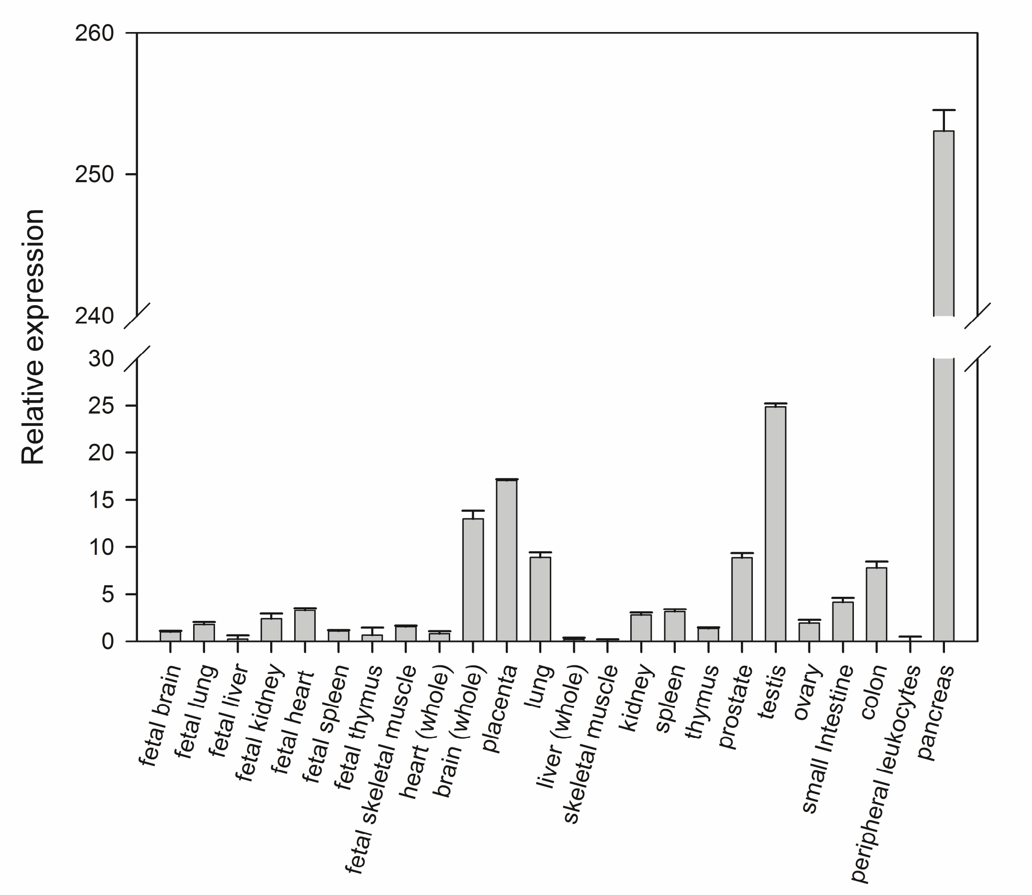
GRM8 (MIM *601116) encoding glutamate receptor, metabotropic 8 is a group III metabotropic glutamate receptor which is linked to the inhibition of the cyclic AMP cascade with high expression in human fetal and adult brains.[15]

CMA showed a 260 kb paternally inherited deletion within the gene *GRM8*. This deletion encompasses exons 8 and 9 of the major isoforms a and b which causes a frame-shift introducing a premature stop codon at aa 516 and resulting in the removal of all transmembrane domains.[15] Concerning the minor, probably secreted isoform c without transmembrane domains,[15] the deletion affects exons 7-9 and causes an addition of 64 aminoacids and stop at amino acid 516 . Sequencing of the gene in the patient for a second hit revealed no mutation.

The boy was born spontaneously at 40 weeks of gestation without complications. Birth weight was 3680 g (50th-75th centile), length 52 cm (25th-50th centile) and head circumference was 34.5 cm (10th-25th centile). Apgar scores were 8/9/10. At the age of 1 year, he was diagnosed with a mixed spastic-ataxic movement disorder and a convergent strabismus with reduced vision on the left eye. He was hypotonic and his development was globally retarded with speech delay and unaided walking at age 3 years. Due to his long face with prominent forehead and periorbital fullness Fragile X syndrome was suspected but molecular testing revealed a normal repeat length. At age 6 10/12 years he showed hypotonia and fine and gross motor clumsiness, but there was no evidence for a movement disorder. He was distractible and hyperactive but was speaking fluently with some deficits and formal Kaufman ABC testing revealed a low normal IQ of about 80 with special strength in the puzzle subtest (level 96). His weight, height, and head circumference were 20.5 kg (19th centile), 121 cm (40th centile) and 49.5 cm (2nd centile), respectively. He received physical, occupational and speech therapy and attended a special school. Physical examination revealed mild left sided strabismus, anteverted nostrils, flat philtrum, large mouth, wide tooth spacing, high-arched palate, retrognathia, clinodactyly of toes 4 and 5 and joint laxity. The older sister who does not carry the deletion also had a mild cerebral movement disorder, low normal head circumference (3rd-10th centile) and low normal cognition with an average HWAIK IV testing result of 88. Their non-carrier mother has had learning disability and developed epilepsy and hearing loss in adulthood. The carrier father attended regular school and completed an apprenticeship in a drug store, but functioned on a low social level.

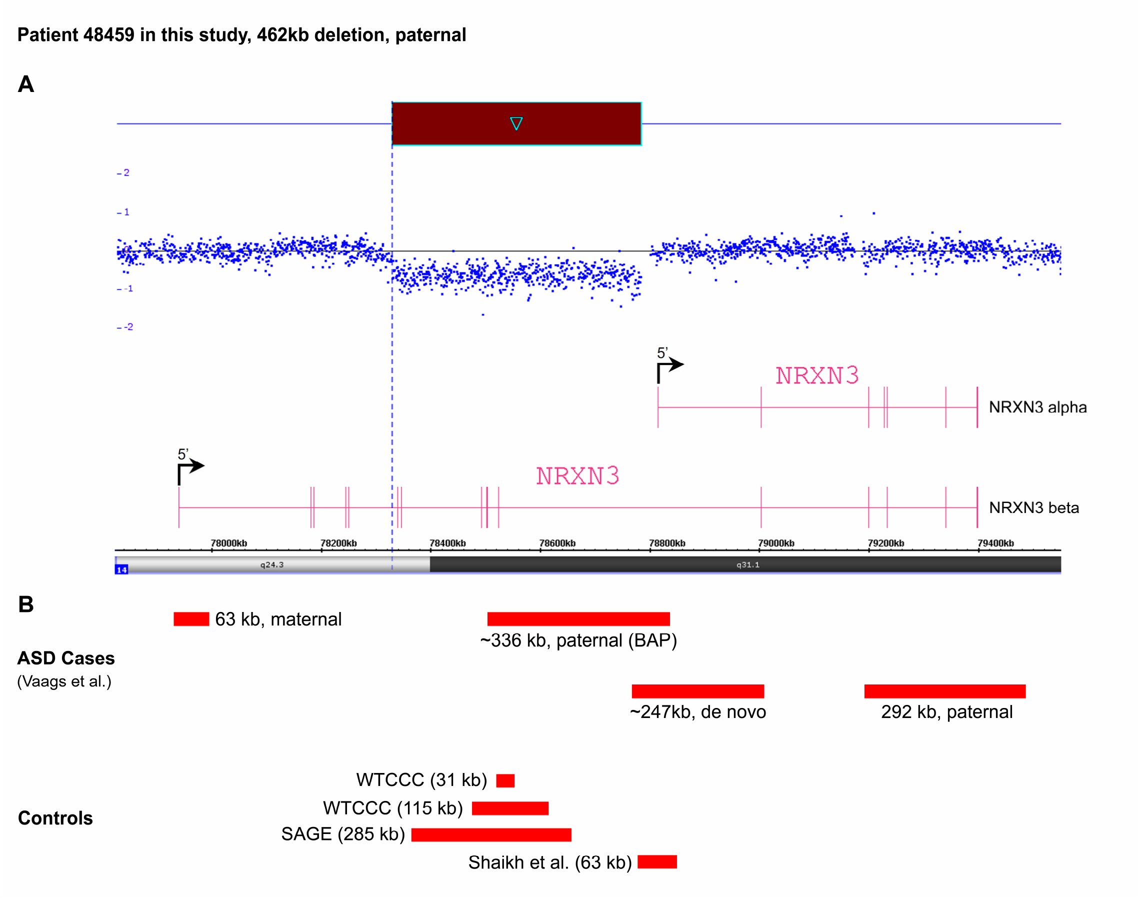
CNVs of glutamate receptor genes have been associated with ADHD including eight patients out of 2493 with deletions in *GRM8* but none in controls.[16] One patient with a paternally inherited 34.7 kb exonic duplication within *GRM8* and two maternally inherited intronic CNVs have been reported among rare CNVs in individuals with ASD.[17] Thus in accordance with the literature findings attention deficit and hyperactivity of the patient could be well explained by *GRM8* defect, but additional unidentified familial factors may contribute to his phenotype.

Figure S1. Expression analysis of *ACOT7* (isoform ENST00000377855) in cDNA panels from fetal and adult human tissues.



Expression analysis was performed using customized SYBR green qPCR for exons 1 & 2 of *ACOT7* (specific for isoform ENST00000377855). Relative expression levels normalized to GAPDH were set into relation to the mean expression value of this isoform in fetal brain. The highest levels were found in adult pancreas, testis, brain, lung, prostate and colon. No expression was detected in peripheral leukocytes.

Figure S2. Screenshot of the array finding in patient 48459 compared to ASD cases reported by Vaags et al.[14] and controls.



(A) Affymetrix 2.7 array derived plots of copy number and log2 ratio values of the *NRXN3* region in patient 48459 indicating a 462 kb deletion encompassing exons 6-12 of the alpha isoform and reaching very closely to the transcription start of the beta isoform. (B) ASD cases with inherited or de novo deletions of *NRXN3* reported by Vaags et al[14] and controls from the Wellcome Trust Case Control Consortium (WTCCC), the Study of Addiction: Genetics and Environment (SAGE) consortium and Shaikh et al.[18] Father of the affected child with the 336 kb paternal deletion has had clinical diagnosis of broader autism phenotype (BAP).

Supplementary Table 2. Clinical and genetic features of patients with inherited heterozygous or homozygous candidate CNVs ≤ 500 kb sorted by descending size

Patient ID	Age* (yrs)	Gender	Phenotype	Aberration	Chromosome band	Genome coordinates	Size (kb)	Confidence value (%)	Marker count	Affected gene(s)	Validation	Sanger sequencing of the affected genes	Pathogenicity
57305	4	M	muscular hypotonia, microcephaly, chorioretinal dysplasia, and lymphedema	duplication	21q21.1	hg18, chr21: 21541563-22029191	488	N/A	331	*NCAM2*	MLPA/ paternally inherited	N/A	likely benign (there is an almost overlapping deletion in Decipher population CNVs (singleton))
48459	7	M	mild learning difficulties, motor problems, facial dysmorphic features, dilated cardiomyopathy, and anal atresia	deletion	14q24.3-q31.1	hg18, chr14: 78321869-78784675)	462	92	586	*NRXN3*	MLPA/ paternally inherited	negative	likely pathogenic, reduced penetrance (a mixture of inherited and de novo deletions within *NRXN3* has been reported in ASD)[14]
64717	6	F	developmental delay, and overgrowth	duplication	16p11.2	hg18, chr16: 29594946-30038054	443	88	287	25 genes on 16p11.2	MLPA/ maternally inherited	N/A	likely pathogenic, reduced penetrance (recurrent microduplication syndrome)[19]
57028	1	F	developmental delay, sever speech delay, microcephaly, facial dysmorphic features, hallux valgus, and short stature	deletion	4q22.3	hg18, chr4: 98745657-99078956	333	93	304	C4orf37 (STPG2)	MLPA/ paternally inherited	negative	VOUS (there are patients 249670 (maternally inherited) similar to our patient and 256952 (inherited from normal parent) with seizure in Decipher database who have intragenic *STPG2* deletions)
53983	1	M	developmental delay, hypotonia, and strabismus	deletion	7q31.33	hg18, chr7: 125890040-126149790	260	93	301	*GRM8*	MLPA/ paternally inherited	negative	likely pathogenic, reduced penetrance (several CNVs of *GRM8* have been reported in ADHD compared to controls)[16]
59700	7	M	global developmental delay, congenital cerebellar ataxia, and sensorineural hearing loss	duplication	19p13.11	hg18, chr19: 17283794-17537339	254	N/A	81	11 genes	MLPA/ maternally inherited	N/A	likely benign (an overlapping duplication is present in 1/1038 of a world wide control cohort by Affymetrix)
60984	4	F	developmental delay, growth hormone deficiency, celiac disease, hypoplastic left kidney	duplication	Xq22.3	hg18, chrX: 110044420-110289813	245	91	301	*PAK3*	MLPA/ paternally inherited	N/A	likely benign (*PAK3* is a known X-linked recessive disease gene. In our case, it is a duplication and it is inherited from the healthy father)
62789	14	F	developmental delay, truncal ataxia, generalized epilepsy, and tall stature	duplication	20p13	hg18, chr20: 2635422-2864768	229	N/A	136	*VPS16*, *FAM113A (PCED1A)*, *C20orf141*, *CPXM1*, *EBF4*, and *PTPRA*	MLPA/- (absent in the mother, but father was N/A, (likely paternal)	N/A	VOUS (this duplication was absent in the mother but present in the likewise affected sister. There are patients 254264 and 272274 in Decipher database with inherited similar duplications and ID)
63153	2	F	developmental delay, hypotonia, epilepsy, and strabismus	duplication	1p34.3	hg18, chr1: 38974598-39179543	205	90	168	*GJA9*, *RRAGC*, *MYCBP*, and *RHBDL2*	MLPA/ paternally inherited	N/A	likely benign (there are three partially overlapping deletions and one duplication in DGV database)
62155	1	M	developmental delay, hypotonia, microcephaly, complex heart defect, short stature, and urether stenosis; follow-up clinical diagnosis of Coffin-Lowry syndrome	deletion	9p22.1	hg18, chr9: 19379757-19555588	176	N/A	113	*ASAH3L (ACER2)*, and *SLC24A2*	MLPA/ maternally inherited	negative	likely benign (pathogenic hemizygous 4 bp del in RPS6KA3 (RSK2): IVS10+4_+7delAGTA was found in this patient confirming the diagnosis of Coffin-Lowry syndrome)

60245	3	F	mild developmental delay, borderline short stature, and peripheral pulmonary stenosis	deletion	16q23.3	hg18, chr16: 81560769-81736858	176	93	259	*CDH13*	MLPA/ maternally inherited	N/A	likely benign (pathogenic heterozygous mutation in PTPN11: c.922A>G (p.N308D) was found in this patient confirming the diagnosis of Noonan syndrome. There are two duplications and five deletions within CDH13 (inherited or undefined) in Decipher database)
55113	18	M	ADHD, microcephaly, short stature, and myopia	deletion	3p14.1	hg18, chr3: 67592633-67762247	170	93	198	*SUCLG2*	MLPA/ maternally inherited	negative	VOUS (there are patients 265229 with inherited intragenic deletion of *SUCLG2* and 259685 with inherited duplication of the whole gene in Decipher database presenting with different phenotypes)
56761	5	M	global developmental delay with prominent speech delay, and cryptorchidism	deletion	Xq28 (pseudoautosomal)	hg18, chrX: 154686877-154843251	156	N/A	15	*VAMP7*	MLPA/ paternally inherited	negative	likely benign (patient 272246 in Decipher database with similar inherited deletion, has an additional 10 Mb de novo deletion. *VAMP7* (*synaptobrevin 1*) has been associated with bipolar affective disorder)[20]
54949	7	M	global developmental delay, muscular hypotonia, ataxia intermittent strabismus convergens, mild thoracic scoliosis, and pes planovalgus,	deletion	6q26	hg18, chr6: 162072914-162156999	84	92	93	*PARK2*	array in parents/ maternally inherited	N/A	VOUS (CNVs of *PARK2* have been shown to be significantly enriched in ASD cases versus controls)[21-22]
50286 and 53032 (siblings)	7.4 and 5	F and M	developmental delay, hypotonia, cleft palate, growth failure, and genital abnormalities (details reported elsewhere)[23]	deletion (homozygous)	2p21	hg18, chr2: 44435674-44519612	83	99	93	*PREPL* and *C2orf34* (*CAMKMT*)	MLPA/ parental	N/A	pathogenic (overlapping Hypotonia Cystinuria- syndrome, described elsewhere)[23]
31553	14	M	autistic features, motor problems, and macrocephaly	deletion	21q22.3	hg18, chr21: 43169759-43201708	32	92	22	*NDUFV3*, and *WDR4*	MLPA/ paternally inherited	negative	VOUS (a de novo missense mutation of *WDR4* has been reported in an autism cohort using WES and mutations of *NDUFV3* have been reported in complex I deficiency)[24-25]
68738	5	M	global developmental delay	deletion	7q35	hg19, chr7: 144509480-144534911	25	93	35	*TPK1*	MLPA/- (absent in the mother, but father	negative	VOUS (this deletion was absent in the mother and a healthy sister but present in the

											was N/A, (likely paternal)		likewise affected sister. A de novo missense mutation of *TPK1* has been reported in an autism cohort using WES)[24]
62611	3	F	developmental delay, muscular hypotonia, and epilepsy	deletion	4q22.1	hg18, chr4: 88438703-88452522	13	90	14	*HSD17B13*	MLPA/ paternally inherited	N/A	likely benign (heterozygous de novo mutation in *SCN2A*: c.4025T>C (p.L1342P) was found in this patient. There are DGV variations 30208 (2/485 ctls), 51479 (3/2026 ctls) and 36311 (1/1 ctl) with deletions encompassing some other exon(s) of *HSD17B13*)
58822	3	M	mild developmental delay, microcephaly, growth deficiency, cryptorchidism, and bilateral inguinal and umbilical hernia	deletion	7q11.22	hg18, chr7: 69233202-69240841	8	93	16	*AUTS2*	MLPA/ paternally inherited	negative	pathogenic (the father is similarly affected. Microdeletions of the gene in patients with variable neuro-developmental features have been reported)[4]
9229	27	M	moderate intellectual disability, epilepsy, and abnormal behaviour	deletion (homozygous)	1p36.31	hg18, chr1: 6361008-6368114 (this position is intronic, however, the nearby exon 1 of *ACOT7* (isoform ENST00000377855) with no markers in the array was confirmed to be deleted by MLPA)	7	98	8	*ACOT7*	MLPA/parental	N/A	likely pathogenic (segregation of the homozygous deletion, function of the gene, its expression pattern, and overlap with the KO mice phenotype are all in favour of its pathogenicity, however, further patients are needed)

* Age at the time of array.

VOUS: variant of uncertain significance, N/A: not available, WES: whole-exome sequencing.

Supplementary Table 3. SNPs of the affected genes in selected patients with inherited heterozygous candidate CNVs ≤ 500 kb detected by Sanger sequencing

Patient ID	**Gender**	**Phenotype**	**Aberration**	**Genome coordinates**	**Size (kb)**	**Affected gene(s)**	**Validation**	**Sanger sequencing of the affected genes**	**Pathogenicity**
48459	M	mild learning difficulties, motor problems, facial dysmorphic features, dilated cardiomyopathy, and anal atresia	deletion	hg18, chr14: 78321869-78784675)	462	*NRXN3*	MLPA/ paternally inherited	1 SNP: c.669C>T (het), p.T 223T, rs1004212, MAF: 0.17	likely pathogenic, reduced penetrance (a mixture of inherited and de novo deletions within *NRXN3* has been reported in ASD)
57028	F	developmental delay, sever speech delay, microcephaly, facial dysmorphic features, hallux valgus, and short stature	deletion	hg18, chr4: 98745657-99078956	333	C4orf37 (STPG2)	MLPA/ paternally inherited	8 SNPs: c.-78T>C (het, 5'UTR), rs4699605, MAF: 0.30 (C) c.222+18a>g (homo), rs13328005, MAF: 0.35 (g) c.373T>C (homo), p.Y125H, rs17558193, MAF: 0.35 (C) c.501-3a>g (homo), rs2903151, MAF:0.35 (g) c.532A>G (homo), p.I178V, rs2903150, MAF: 0.35 (g) c.557A>G (het), p.Y186C, rs28403003, MAF: 0.05 (G) c.579A>G (homo), p.L193L, rs2865979, MAF: 0.35 (G) c.1204+16c>t (het), rs202103504, MAF: < 0.01 (t)	VOUS (there are patients 249670 (maternally inherited) similar to our patient and 256952 (inherited from normal parent) with seizure in Decipher database which have intragenic STPG2 deletions)
53983	M	developmental delay, hypotonia, and strabismus	deletion	hg18, chr7: 125890040-126149790	260	*GRM8*	MLPA/ paternally inherited	2 SNPs: c.1018+14A>G (het), rs769199, MAF: 0.04 (A) c.31T>C (het, 3' UTR), rs712723, MAF: 0.41	likely pathogenic, reduced penetrance (several CNVs of *GRM8* have been reported in ADHD compared to controls)
62155	M	developmental delay, hypotonia, microcephaly, complex heart defect, short stature, and urether stenosis; follow-up clinical diagnosis of Coffin-Lowry syndrome	deletion	hg18, chr9: 19379757-19555588	176	*ASAH3L (ACER2)*, and *SLC24A2*	MLPA/ maternally inherited	2 SNPs in *SLC24A2*: c.960A>G (het), p.P320P, rs4977308, MAF: 0.21 (A) c.1201A>C (het), p.R401R, rs2383101, MAF: 0.15 (A)	likely benign (pathogenic hemizygous 4 bp del in RPS6KA3 (RSK2): IVS10+4_+7delAGTA was found in this patient confirming the clinical diagnosis of Coffin-Lowry syndrome)
55113	M	ADHD, microcephaly, short stature, and myopia	deletion	hg18, chr3: 67592633-67762247	170	*SUCLG2*	MLPA/ maternally inherited	1 SNP: c.45G>A (homo, 3' UTR), rs1065399, MAF: 0.32 (G)	VOUS (there are patients 265229 with inherited intragenic deletion of SUCLG2 and 259685 with inherited duplication of the whole gene in Decipher database presenting with different phenotypes)
56761	M	global developmental delay with prominent speech delay, and cryptorchidism	deletion	hg18, chrX: 154686877-154843251	156	*VAMP7*	MLPA/ paternally inherited	1 SNP: c.595-10G>C (homo), rs143821247, MAF: 0.310	likely benign (patient 272246 in Decipher database with similar inherited deletion, has an additional 10Mb de novo deletion. *VAMP7* (*synaptobrevin 1*) has been associated with bipolar affective disorder)

31553	M	autistic features, motor problems, and macrocephaly	deletion	hg18, chr21: 43169759-43201708	32	*NDUFV3*, and *WDR4*	MLPA/ paternally inherited	3 SNPs in *WDR4*: c.213G>C (het), p.K71N, rs2248490, MAF: 0.494 (G) c.796C>T (het), p.P266S, rs15736, MAF: 0.35 c.1169G>A (het), p.R390Q, rs6586250, MAF: 0.2	VOUS (a de novo missense mutation of *WDR4* has been reported in an autism cohort using WES and mutations of *NDUFV3* have been reported in complex I deficiency)
68738	M	global developmental delay	deletion	hg19, chr7: 144509480-144534911	25	*TPK1*	MLPA/- (absent in the mother, but father was N/A, (likely paternal)	-	VOUS (this deletion was absent in the mother and a healthy sister but present in the likewise affected sister. A de novo missense mutation of *TPK1* has been reported in an autism cohort using WES)
58822	M	mild developmental delay, microcephaly, growth deficiency, cryptorchidism, and bilateral inguinal and umbilical hernia	deletion	hg18, chr7: 69233202-69240841	8	*AUTS2*	MLPA/ paternally inherited	1 silent variant: c.1158C>T (het), p.S386S	pathogenic (the father is similarly affected. Microdeletions of the gene in patients with variable neurodevelopmental features have been reported)
60984	F	developmental delay, growth hormon deficiency, left hypoplastic kidney, and celiac disease	deletion	hg18, chr16: 24793142-24797112	3	*SLC5A11*	MLPA/ maternally inherited	1 SNP: c.669T>C (homo), p.F223F, rs274081, MAF: <0.01 (T)	no evidence (many members of Solute Carrier Family are involved in genetic disorders)

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