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ARTICLE

Rare copy number variation in cerebral palsy

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Recent studies have established the role of rare copy number variants (CNVs) in several neurological disorders but the contribution of rare CNVs to cerebral palsy (CP) is not known. Fifty Caucasian families having children with CP were studied using two microarray designs. Potentially pathogenic, rare (<1% population frequency) CNVs were identified, and their frequency determined, by comparing the CNVs found in cases with 8329 adult controls with no known neurological disorders. Ten of the 50 cases (20%) had rare CNVs of potential relevance to CP; there were a total of 14 CNVs, which were observed in <0.1% (<8/8329) of the control population. Eight inherited from an unaffected mother: a 751-kb deletion including *FSCB*, a 1.5-Mb duplication of 7q21.13, a 534-kb duplication of 15q11.2, a 446-kb duplication including *CTNND2*, a 219-kb duplication including *MCPH1*, a 169-kb duplication of 22q13.33, a 64-kb duplication of *MC2R*, and a 135-bp exonic deletion of *SLC06A1*. Three inherited from an unaffected father: a 386-kb deletion of 12p12.2-p12.1, a 234-kb duplication of 10q26.13, and a 4-kb exonic deletion of *COPS3*. The inheritance was unknown for three CNVs: a 157-bp exonic deletion of *ACOX1*, a 693-kb duplication of 17q25.3, and a 265-kb duplication of *DAAM1*. This is the first systematic study of CNVs in CP, and although it did not identify *de novo* mutations, has shown inherited, rare CNVs involving potentially pathogenic genes and pathways requiring further investigation.
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INTRODUCTION

Cerebral palsy (CP) is the most common motor disorder of childhood, with a prevalence of 2–2.5/1000 live births.[1,2] CP has been defined as 'a group of permanent disorders of the development of movement and posture, causing activity limitation, which are attributed to non-progressive disturbances that occurred in the developing foetal or infant brain. The motor disorders of CP are often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour by intellectual disability, autism and epilepsy, and by secondary musculoskeletal problems.[3] Evidence of intrapartum foetal compromise is found at birth in <10% of cases.[4] There are a number of known major epidemiological risk factors for CP, including preterm delivery, intrauterine growth restriction (IUGR), intrauterine infection, and multiple pregnancy.[5]

There is evidence of genetic susceptibility to CP but little is known about possible mechanisms.[6] Monozygotic twins have a significantly higher ($P=0.0026$) concordance rate for CP compared with dizygotic twins.[7] Several family studies have determined the likely inheritance pattern responsible for different clinical manifestations of CP.[8,9] SNP association studies have been reported but have provided inconclusive results because of small sample sizes.[10]

A genetic linkage study mapped an autosomal recessive type of spastic CP to a locus on chromosome 2p24-25[11] and subsequently, a missense variant was found in the *GAD1* gene, which segregated with the CP phenotype (LOD score 5.75).[12] However, the functional effect of this variant was not assessed and no *GAD1* variants have since been found in other individuals with CP. In another genetic linkage study, ataxic CP affecting four children from a complex consanguineous family was mapped to chromosome 9p12-q12 (LOD score 3.4).[13]

More recently, mutations in all four subunits of the adaptor protein complex-4 (*AP4E1*, *AP4M1*, *AP4B1*, and *AP4S1*) were reported to cause a CP-like motor disorder.[14–17] The phenotype of the affected individuals included slowly progressive spasticity, and therefore does not fit the classical definition of CP, which assumes non-progression. A very slowly progressive neurological disorder may be classified as CP at one point in time, with the need to change the diagnosis after a longer period of observation.[18] It is now clear that *de novo* and inherited copy number variants (CNVs) contribute to the aetiology of various neurological disorders, such as autism,[19] intellectual disability,[20,21] and epilepsy.[22] The extent to which this is based on single 'hits' of large effect, or two or more hits of smaller effect acting synergistically, is uncertain.[20,23] The contribution of CNVs to CP has not yet been systematically investigated; this work addresses this knowledge gap by testing a selected cohort of 50 cases with CP using two customised microarray platforms.

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METHODS

DNA samples and study cohort

To determine whether rare CNVs (<1% population frequency) contribute to CP causation, we used two separate custom microarrays to study DNA samples from 50 Caucasian children with a diagnosis of CP confirmed by a specialist. All cases were identified from the South Australia CP register and recruited through paediatric rehabilitation specialists at the Women's and Children's Hospital, Adelaide.[18] Parents were tested when potentially relevant CNVs were found and parental samples were available. No other selection criteria were applied. Clinical data were collected for each case, including gender, details of pregnancy and birth, gestational age, Apgar scores, birth weight, head circumference, and neonatal events. Also collected, were type of CP and comorbidities such as intellectual disability, autism, and epilepsy. Growth restriction was assessed using a customised birth weight centile programme.[24]

Results were compared with 8329 samples from adults with no known neurological disorders.[21] This control data set has been described previously in detail.[21] Results were also compared with the frequency of events in 337 National Institute of Mental Health (NIMH) controls analysed using the same 135K array.[25]

DNA isolated from lymphoblastoid cell lines was used for CNV analyses, and DNA isolated from whole blood was used for subsequent CNV validations. Parental DNA was isolated from whole blood only. Signed parental consent and ethics approval from the Adelaide Women's and Children's Health Network Human Research Ethics Committee were obtained.

CNV discovery

Two customised microarray platforms were used. The first was a customised 180K chromosomal microarray with targeted plus whole-genome coverage (Agilent Technologies, Santa Clara, CA, USA). The targeted coverage included clinically relevant regions such as known deletion/duplication syndrome regions, telomeres, and centromeres at a resolution of ~20–50 kb plus exon-level coverage of >1200 genes involved in neurodevelopmental disorders. The whole-genome backbone results in a resolution in unique DNA of ~225 kb. Feature Extraction (version 10.5.1.1) and DNA Analytics (Version 4.0.81) software (Agilent Technologies) was used to perform data analysis.

The second array platform was a customised 135K microarray (Roche NimbleGen, Madison, WI, USA), with one probe every 2.5 kb in the genomic hotspots (regions flanked by segmental duplications) and 35 kb density in the genomic backbone. All microarray hybridisation experiments were performed as described previously,[26] using a single unaffected male (GM15724 from Coriell, Camden, NJ, USA) as reference. The empirically determined detection resolution for this array was >50 kb within the hotspots and >350 kb in the genomic backbone.[25] All microarrays were analysed by mapping probe coordinates to the human genome assembly Build 36 (hg18). To minimise false positives on both array platforms, deletions and duplications were determined by a minimum of four consecutive probes beyond a mean significance log 2 ratio of −0.26 and 0.3, respectively. All CNV calls were manually inspected by loading the signal intensity data onto a custom University of California Santa Cruz browser. CNV regions of interest identified in CP cases and their parents were validated by quantitative real-time PCR (qPCR) (Supplementary Table 1).

RESULTS

Clinical details of cases with rare CNVs

We studied 50 cases of CP, all born to non-consanguineous parents of Caucasian (northern European) descent. There were 28 males and 22 females. Twenty-four (48%) were born at term and the remaining 26 (52%) were born at <37 weeks gestation. The mean birth weight was 2352 g (680–4650 g). One out of the 50 cases (2%) had documented IUGR. Spastic hemiplegia was the most prevalent type of CP with 22 cases (44%), followed by diplegia with 13 cases (26%), quadriplegia with 13 cases (26%), and triplegia with 2 cases (4%). The gross motor function classification system (GMFCS) was used to measure severity: level 1, which indicated independent walking through to level 5 where the individual is wheelchair-dependent.[27] Twelve cases (24%) were documented at level 1, 8 cases (16%) at level 2, 4 cases (8%) at level 3, 5 cases (10%) at level 4, 8 cases (16%) at level 5, and for 13 cases GMFCS was not documented. Sixteen cases (32%) had intellectual disability, 16 cases (32%) had epilepsy, and autism was reported in 5 cases (10%). The mean maternal and paternal ages were 30 years (22–42 years) and 32 years (23–47 years), respectively, (Supplementary Table 2).

CNV burden in CP

We assessed the total CNV load in individuals with CP ($n=50$) compared with those from previously published CNV data on autism ($n=350$), intellectual disability ($n=501$), and controls from the NIMH cohort.[25] Notably, all these CNVs were discovered using the same array design facilitating comparison across disease cohorts. CNV load in CP was similar to that in controls or the dyslexia cohort (Figure 1).

Rare CNV discovery and validation

For rare CNV detection, we utilised CNV calls from both array platforms (see Methods). Eighty-four variants were identified by using both arrays, therefore considered validated. On the basis of comparison with CNV calls from population controls these CNVs were classified as rare, potentially pathogenic events, benign, or common variants (>1% population frequency; Supplementary Table 3).

Fourteen different rare (observed in <0.1% (<8/8329) of the control population) potentially pathogenic CNVs found in 10 out of the 50 cases (20%) were studied.[21] Their mean genomic size was 355 kb (range 135 bp–1.5 Mb). There were five (36%) deletions with mean size 228 kb (135 bp–741 kb), and nine (64%) duplications with mean size of 466 kb (65 kb–1.5 Mb). None of these rare potentially pathogenic variants were observed among the NIMH controls, but the small sample sizes precludes statistical rigour (Table 1).

Eight of the 14 CNVs were maternally inherited. Five involved single genes: a 751-kb deletion involving the entire *FSCB* gene, a 446-kb duplication that included the first exon of *CTNND2*, a 219-kb duplication involving the first exon of *MCPH1*, and a 64-kb duplication involving the entire *MC2R* gene and a 135-bp exonic deletion involving *SLC06A1*. The other three maternally inherited CNVs contained several genes each: a 1.5-Mb duplication of 7q21.13 (*ZNF804B, MGC26647, STEAP1, STEAP2, FLJ21062*), a 535-kb

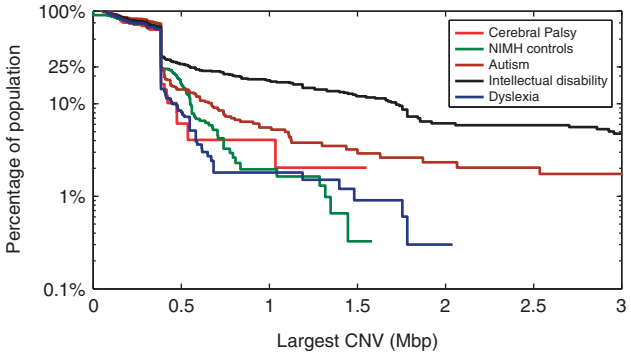

Figure 1 CNV burden analysis for CP. The figure shows the population frequency of the largest CNV (as a survivor function) in individuals with CP compared with 337 controls from the NIMH cohort and as a comparison of CNV burden to CNV data from individuals with intellectual disability, autism, and dyslexia (data from Girirajan *et al*.[25]).

Table 1 Summary of CNVs in 10 cases

Case	Cytoband 180K array Build 36 (hg 18)	Gene position	Size (bp)	CNV	Gene(s)	Inheritance	135K array Build 36 (hg 18)	qPCR	Controls [a]Controls (8329)	Controls NIMH (337)	Sex	Gestation (weeks)	HC	Subtype
P003	7q21.13	Chr7:88 152 747–89 720 583	1 567 836	dup	*ZNF804B, MGC26647, STEAP1,*	Maternal	Yes	Yes	4	NA	F	32	30	Dip
	12p12.2-p12.1	Chr12:20 908 843–21 295 433	386 590	del	*STEAP2, STEAP2, STEAP2, FLJ21062, SLCO1B3, LST-3TM12, SLCO1B1*	Paternal	No	Yes	2	NA				
P011	14q23.1	Chr14:58 593 026–58 804 264	265 283	dup	*DAAM1*	Unknown	No	Yes	0	0	F	30	28	Dip
P013	17q25.3	Chr17:74 722 430–75 415 451	693 021	dup	*HRNBP3, ENPP7, CBX2, CBX2, CBX8*	Unknown	Yes	Yes	0	NA	F	40	U	Hem
P017	14q21.3	Chr14:43 617 568–44 369 205	751 637	del	*FSCB*	Maternal	Yes	Yes	1	0	M	39	U	Quad
P020	17p11.2	Chr17:17 104 399–17 108 906	4507	del	*COPS3* intragenic	Paternal	No	Yes	NA	NA	F	40	U	Hem
P023	17q25.1	Chr17:71 467 868–71 468 025	157	del	*ACOX1* exonic	Unknown	No	Yes	NA	NA	F	39	33.5	Quad
P025	15q11.2	Chr15:20 316 992–20 851 728	534 736	dup	*TUBGCP5, TUBGCP5, CYFIP1, CYFIP1,*	Maternal	Yes	Yes	35	3	M	40	35	Hem
	8p23.1-p23.2	Chr8:6 072 234–6 291 658	219 424	dup	*NIPA2, NIPA1, MCPH1* first exon	Maternal	No	Yes	0	0				
P036	5p15.2	Chr5:11 956 988–12 403 682	446 694	dup	*CTNND2* first exon	Maternal	Yes	Yes	0	0	F	41	33	Hem
P053	18p11.21	Chr18:13 861 404–13 925 908	64 504	dup	*MC2R* entire gene	Maternal	No	Yes	0	0	M	36	35	Quad
	22q13.33	Chr22:49 154 182–49 323 370	169 188	dup	*SAPS2, SBF1, ADM2, MIOX, LMF2, NCAPH2,*	Maternal	No	Yes	1	NA				
	5q21.1	Chr5:101 763 193–101 763 328	135	del	*NCAPH2, SCO2, TYMP, TYMP, LOC440836, SLCO6A1* exonic	Maternal	No	Yes	NA	NA				
P057	10q26.13	Chr10:126 466 575–126 700 900	234 325	dup	*METTL10, KIAA0157, ZRANB1, CTBP2*	Paternal	No	Yes	0	NA	M	29	U	Hem

Abbreviations: Dip, diplegia; HC, head circumference; Hem, hemiplegia; NA, denotes no data on controls due to low probe coverage; NIMH, National Institute of Mental Health; qPCR, quantitative real-time PCR; Quad, quadriplegia; U, no available data.
[a]8329 adult controls with no known neurological disorders.

duplication of 15q11.2 (*TUBGCP5, CYFIP1, NIPA2, NIPA1*), and a169-kb duplication of 22q13.33 (*SAPS2, SBF1, ADM2, MIOX, LMF2, NCAPH2, SCO2, TYMP, LOC440836*). Three of the 14 CNVs were paternally inherited. One involved a single gene; a 4-kb deletion including exons 6–8 of the *COPS3* gene. The other two paternally inherited CNVs contained several genes each: a 387-kb deletion on 12p12.2-p12.1 (*SLCO1B3, LST-3TM12, SLCO1B1*) and a 234-kb duplication of 10q26.13 (*METTL10, KIAA0157, ZRANB1, CTBP2*). Of these 11 CNVs, 2 (12p12.2-p12.1 and *COPS3*) were inherited from father to daughter, and 6 (*MCPH1*, *FSCB*, *MC2R*, *SLCO6A1*, 15q11.2, and 22q13.33) were inherited from mother to son. We could not determine whether the remaining three CNVs were inherited or *de novo* due to the unavailability of parental DNA. These included two single-gene CNVs: a 157-bp exonic deletion of *ACOX1* and a 265-kb duplication of exons 1–4 of *DAAM1*, and one CNV containing several genes: a 693-kb duplication of 17q25.3 (*HRNBP3, ENPP7, CBX2, CBX8*). Five of the 14 CNVs were identified by both arrays, 9 were found on the 180K array only, as the 135K array did not have sufficient probe coverage for these regions. Each of these fourteen CNVs was validated by qPCR (Table 1).

Several of these CNVs involve known OMIM (Online Mendelian Inheritance in Man) genes. These included *CTNND2* (OMIM 604275), *MCPH1* (OMIM 607117, 251200), *NIPA2* (OMIM 608146), *NIPA1* (OMIM 608145), *ACOX1* (OMIM 264470), and *MC2R* (OMIM 607397). Four of these genes are also reported in Decipher as having a predicted likelihood of haploinsufficiency (HI): *CTNND2* (26.1%), *NIPA1* (25.3%), *COPS3* (19.5%), and *DAAM1* (8.2%).

DISCUSSION

This is the first study to assess the potential contribution of CNVs to the aetiology of CP. Two different customised microarray platforms were utilised to maximise CNV detection in this cohort. Both platforms had been designed and validated for clinical testing of disorders found in high prevalence in the population, including intellectual disability, autism, and epilepsy, disorders that are common co-morbidities of CP. We identified 14 CNVs that are plausible contributors to the aetiology of CP in 10 of 50 (20%) affected individuals. All are rare (<1% population frequency). Ten of the 14 CNVs were larger than 150 kb. Five of the 14 CNVs were deletions with an average size of 228 kb and 1 of them involved a gene, *COPS3* that is predicted to be dosage sensitive. Nine of the 14 CNVs were duplications with an average size of 466 kb (the largest spanning

1.5 Mb), and 3 involved genes (*CTNND2, NIPA1,* and *DAAM1*) that are predicted to be dosage sensitive.

All 14 CNVs involve genes expressed in the brain, several of which have been reported to participate in the pathogenesis of other neurological disorders. These include: (i) *ACOX1* 157 bp exonic deletion found in case P023. *ACOX1* is the first enzyme in the peroxisomal β-oxidation very-long-chain fatty acids. Autosomal recessive mutations in *ACOX1* cause peroxisomal acyl-CoA oxidase deficiency (OMIM 264470) and involve the nervous system. Clinical phenotypes with *ACOX1* deficiency include white matter abnormalities, early onset hypotonia, neonatal seizures, and psychomotor delay.[28] (ii) A small 4-kb deletion of exons 4–6 of *COPS3* in case P020. *COPS3* is one of eight subunits of the COP9 signalosome protein complex, and is involved in a variety of cellular and developmental processes and in signal transduction.[29] Previous studies have shown that lack of any of the eight subunits of COP9 can destabilise the structure of the entire complex.[30] *COPS3* maps to chromosome 17p11.2, within the Smith-Magenis syndrome (SMS) and Potocki-Lupski syndrome (PTLS) critical interval.[31,32] Autism spectrum disorder, the main feature of PTLS, has been reported in $\sim$80% of cases.[33] As with SMS, PTLS is also characterised by congenital abnormalities and intellectual disability.[33] (iii) A 534-kb duplication on 15q11.2 found in case P025 includes four highly conserved genes: *TUBGCP5, CYFIP1, NIPA2,* and *NIPA1*. *TUBGCP5* is expressed in the subthalamic nuclei and *CYFIP1* is widely expressed in the central nervous system. Deletions of 15q11.2 map to the critical region of the Prader-Willi syndrome/Angelman syndrome[34] (OMIM 608145, 608146). Microdeletions of 15q11.2 region have also been reported in cases with delayed motor and speech development, autism, obsessive-compulsive disorder, and dysmorphic features without Prader-Willi/Angelman syndrome, suggesting HI for *TUBGCP5, CYFIP1, NIPA2,* and *NIPA1*.[35] Although observed in control individuals, the role of 15q11.2 duplications is yet to be unravelled. (iv) *CTNND2* 446 bp duplication in case P036. *CTNND2* is highly expressed in foetal brain and has an important role in neuronal functioning, adhesion, and migration.[36,37] It is involved in early embryogenesis and encodes an adhesive junction-associated protein of the armadillo/β-catenin superfamily.[38] *CTNND2* has been associated with intellectual disability in cri-du-chat syndrome[36] (OMIM 604275), autism when deleted,[38,39] and schizophrenia when duplicated.[37] Previous studies have reported both dosage variation and disruptive effects resulting from CNVs involving *CTNND2*.[37,40] This gene has a predicted HI of 26.1%. (v) A 219-bp duplication in case P025 involving exon 1 of *MCPH1*. Microcephalin, the product of *MCPH1,* highly expressed in the foetal cerebral cortex[41] is involved in neurogenesis and regulation of cerebral cortex size. Homozygous loss of function mutations of the *MCPH1* gene (OMIM 607117, 606858, and 251200) cause autosomal recessive disorders including premature chromosome condensation syndrome,[42] intellectual disability,[42] and microcephaly.[43] Heterozygous deletions and duplications of *MCPH1* have been reported in families with autism spectrum disorders, supporting the concept that *MCPH1* is a dosage-sensitive gene, with considerable mutation pleiotropy.[44] (vi) A 64-kb duplication of *MC2R* identified in case P053. *MC2R* can result in the rare autosomal recessive disorder, familial glucocorticoid deficiency. Clinical characteristics related to hypoglycaemia can include hypertonic seizures, skin hyperpigmentation, and muscle weakness.[45]

Eleven of the 14 CNVs that we identified were inherited from an unaffected parent and were considered to be of potential relevance to CP. Reasons for discordance between parent and child could include variable expressivity,[46] incomplete penetrance,[47] and epigenetic modification of gene expression.[47] Expression of a clinical phenotype in the child but not in the parent could also be explained by the 'two hit' hypothesis,[20,23] which postulates the additive effects of two or more *de novo* or inherited genetic abnormalities in the affected individual, with fewer such abnormalities in the unaffected parent. Other mechanisms include the presence of a point mutation on the other allele in the affected children, not identified by the CNV profiling,[48] and various combinations of CNVs and point mutations affecting different genes.[23] Genetic susceptibility may also be triggered by environmental risk factors for CP such as prematurity and IUGR.[5]

In some cases, it will be the combination of genetic and non-genetic risk factors that trigger the causal pathway to CP. Four out of our 10 cases were born prematurely and 2 of these carried two or more CNVs. Another case, born at term, carried two maternally inherited CNVs: including a 535-kb duplication of 15q11.2 and a 219-kb duplication of *MCPH1*. Homozygous mutations involving *MCPH1* are known to cause autosomal recessive primary microcephaly. In this case microcephaly was absent, head circumference was normal at birth (35 cm) and again at 7 months of age (45 cm). Magnetic resonance imaging showed left porencephaly following periventricular leukomalacia, which would be atypical of microcephaly. Sequencing of the other *MCPH1* allele showed no other variant (unpublished data). Another case was born following an emergency caesarean section, performed because of non-reassuring foetal condition and diagnosed with IUGR and hypothyroidism. However, there did not appear to be a difference in perinatal risk factors between the 10 cases with CNVs and the other 40 cases in the cohort. Type and severity of CP and other comorbidities were also similar between these two groups.

The strengths of this initial study are the use of two array platforms, the design and criteria for CNV calling, the rigorous validation and the plausible role these individual CNVs might have in disrupting various neurodevelopmental pathways.

The weaknesses of this study are that no *de novo* CNVs were found, and it remains to be determined whether the inherited CNVs described led to a CP phenotype due to co-genetic or environmental triggers. The small sample size and the inherited nature of the CNVs make it difficult to determine their true pathogenicity. The cases collected for this study were by necessity from a heterogeneous group of volunteer families, which was generally representative of the CP population in terms of gestational age, risk factors for CP, and CP subtype. No exclusions were made on the grounds of preconceived assumptions as to causation. Although the CNVs described may be involved in plausible biological pathways, one of the two arrays was intentionally targeted at genes known to be involved in neurodevelopmental disorders. This potential bias was offset by the rarity of these events in the control population. Nevertheless, their precise contribution to CP requires much more investigation.

Not all parents were tested and of the cases whose parents were tested, no *de novo* CNVs were identified at the resolution of the 180K array (20–50 kb-targeted regions, 225 genomic backbone) and the 135K array ($>$50 kb hotspot region, $>$50 kb genomic backbone). An increased CNV burden was not observed compared with the NIMH control cohort. This may reflect the lack of statistical power in detecting rare events in this cohort, or could be due to the fact that both array platforms were underpowered for detecting genome-wide small CNVs. Therefore, the true CNV contribution to CP may be underestimated. There was potential bias in our study cohort with regards to subtype and severity of CP. Only 13 out of 50 cases (26%) had a GMFCS score of $\geq$4. It is possible that *de novo* pathogenic

CNVs will have an increased yield in individuals with more severe CP subtypes. We identified 10 out of 50 cases with one or more potentially pathogenic CNVs for CP. Where parental DNA was available, 11 out of 14 CNVs were found to be inherited from an unaffected parent, suggesting a more complex genetic aetiology than a major causative effect. The contribution of other genetic or environmental factors to CP in this cohort was not investigated. Genome/exome sequencing has the potential to identify further sequence variation, which likely contributes to CP and was not evaluated in this study. Evaluation of the contribution of CNVs to CP awaits further confirmation in a larger independent cohort. However, these data suggest that in this cohort CNVs may contribute to the pathogenicity of CP in up to 20% of cases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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WEB RESOURCES

Electronic-database information: Gestation Network (http://www.gestation.net): University of California Santa Cruz (UCSC) browser: http://www.genome.ucsc.edu/cgi-bin/hgGateway.: Online Mendelian Inheritance in Man (http://www.omim.org): Decipher –Wellcome Trust Sanger Institute (https://www.decipher.sanger.ac.uk).

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