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Clinical relevance of small copy-number variants in chromosomal microarray clinical testing

Dana Hollenbeck, MPH, MS[1], Crescenda L. Williams, MS[1,2], Kathryn Drazba, MPH, MS[1], Maria Descartes, MD[1,3], Bruce R. Korf, MD, PhD[1,3], S. Lane Rutledge, MD[1,3], Edward J. Lose, MD[1,3], Nathaniel H. Robin, MD[1,3], Andrew J. Carroll, PhD[1] and Fady M. Mikhail, MD, PhD[1]

Purpose: The 2010 consensus statement on diagnostic chromosomal microarray (CMA) testing recommended an array resolution ≥400 kb throughout the genome as a balance of analytical and clinical sensitivity. In spite of the clear evidence for pathogenicity of large copy-number variants (CNVs) in neurodevelopmental disorders and/or congenital anomalies, the significance of small, nonrecurrent CNVs (<500 kb) has not been well established in a clinical setting.

Methods: We investigated the clinical significance of all nonpolymorphic small, nonrecurrent CNVs (<500 kb) in patients referred for CMA clinical testing over a period of 6 years, from 2009 to 2014 (a total of 4,417 patients). We excluded from our study patients with benign or likely benign CNVs and patients with only recurrent microdeletions/microduplications <500 kb.

Results: In total, 383 patients (8.67%) were found to carry at least one small, nonrecurrent CNV, of whom 176 patients (3.98%) had one small CNV classified as a variant of uncertain significance (VUS), 45 (1.02%) had two or more small VUS CNVs, 20 (0.45%) had one small VUS CNV and a recurrent CNV, 113 (2.56%) had one small pathogenic or likely pathogenic CNV, 17 (0.38%) had two or more small pathogenic or likely pathogenic CNVs, and 12 (0.27%) had one small pathogenic or likely pathogenic CNV and a recurrent CNV. Within the pathogenic group, 80 of 142 patients (56% of all small pathogenic CNV cases) were found to have a single whole-gene or exonic deletion. The themes that emerged from our study are presented in the Discussion section.

Conclusions: Our study demonstrates the diagnostic clinical relevance of small, nonrecurrent CNVs <500 kb during CMA clinical testing and underscores the need for careful clinical interpretation of these CNVs.

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Key Words: CMA clinical testing; intragenic deletion; single-gene deletion; small CNV

INTRODUCTION

Cytogenetic testing has long been used to identify the loss or gain of genetic material. High-resolution chromosomal microarray (CMA) has emerged as the gold standard for identifying small losses or gains of genomic material, also called copy-number variants (CNVs), which could be implicated in the pathogenesis of developmental delay, intellectual disability, autism spectrum disorders, and/or congenital anomalies.[1] In 2010 CMA was designated as a first-tier clinical diagnostic test for individuals with neurodevelopmental disorders and congenital anomalies. This testing raised the diagnostic yield of cytogenetic testing from 4 to 17–19%.[2] The CMA technology has evolved from utilizing targeted areas of known recurrent microdeletions and microduplications to a platform that also includes whole-genome coverage capable of identifying novel microdeletions and microduplications anywhere in the human genome.[3] CMA technology is able to identify abnormalities as small as 20–50 kb in targeted regions and 100–250 kb throughout the genome.[2] This level of resolution allows for the detection of abnormalities spanning single genes as well as intragenic CNVs, thus raising the diagnostic yield of the test. Higher resolution platforms, however, also lead to the increased detection of CNVs with uncertain clinical significance.[2,4] Interpretation of these rare CNVs is further complicated by the presence of CNVs in healthy individuals (benign CNVs).[5] In an effort to unify clinical interpretation, the 2010 consensus statement on diagnostic CMA testing was released, which recommended an array resolution ≥400 kb throughout the genome as a balance of analytical and clinical sensitivity; however, the American College of Medical Genetics and Genomics standards and guidelines encouraged individual laboratories to determine their own size restrictions.[2,6] The clinical significance of small, nonrecurrent CNVs (<500 kb) that are not mediated by nonallelic homologous recombination has not been well established in a clinical setting. Recent efforts have been made to understand the role of these small, nonrecurrent CNVs in neurodevelopmental disorders.[7–9]

In this study we evaluate the role of these small, nonrecurrent CNVs (<500 kb) in a cohort of 4,417 patients referred to our lab to determine the overall significance of these CNVs in patients undergoing CMA clinical testing for developmental

[1]Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA; [2]Current address: Children's Health Hospital, Dallas,Texas, USA; [3]Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama, USA. Correspondence: Fady M. Mikhail (fmikhail@uab.edu)

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delay, intellectual disability, dysmorphic features, and/or congenital anomalies.

MATERIALS AND METHODS

Patients

Genotype and phenotype information were collected for all patients who were referred for CMA clinical testing at the University of Alabama at Birmingham Clinical Cytogenetics Laboratory during the period between 2009 and 2014, and had an abnormal CMA result. Phenotypic information was collected using a standardized phenotypic checklist utilizing Human Phenotype Ontology codes (**Supplementary Table S1** online). These data have been collected for all patients with abnormal CMA results as part of our ongoing effort to assemble a comprehensive genotype–phenotype database. Inclusion criteria in this study included identification of a small, nonrecurrent CNV (<500 kb) that is not mediated by nonallelic homologous recombination without the presence of a larger (>500 kb) pathogenic abnormality. Individuals were excluded from our study if the identified abnormalities were classified as benign or likely benign CNVs. We also excluded recurrent microdeletions/microduplications <500 kb that are mediated by nonallelic homologous recombination, unless they coexisted with a small, nonrecurrent CNV. This study was approved by the institutional review board for human subject research at the University of Alabama at Birmingham.

Methods

High-resolution whole-genome array comparative genomic hybridization (aCGH) was performed using three platforms. The Agilent 4×44k aCGH, Agilent 8×60k aCGH, and Agilent 4×180k aCGH+SNP platforms were used from January 2009 through December 2011, January 2012 through August 2013, and September 2013 through December 2014, respectively (Agilent Technologies, Santa Clara, CA). All three platforms are custom-designed arrays that are based on the International Standard Cytogenomic Array Consortium design. Peripheral blood samples were used for DNA extraction. A more detailed description of aCGH methodology is available in the **Supplementary Material** online. Genomic breakpoints for patients analyzed using the 4×44k and 8×60k aCGH platforms were mapped using the UCSC Genome Browser human genome build 36 (NCBI36/hg18). Genomic breakpoints for patients analyzed using the 4×180k aCGH+SNP platform were mapped using the UCSC Genome Browser human genome build 37 (GRCh37/hg19).

The reporting criteria for deletions required a minimum size of >50–100 kb within the backbone coverage and >5–10 kb within a targeted, well-characterized haploinsufficient gene. For duplications and triplications, the reporting criteria required a minimum size of >200 kb within the backbone coverage or smaller if it spans a well-characterized triplosensitive gene or one or both breakpoints map within a clinically relevant gene. Our cutoffs for CNV calls are three adjacent oligos (probes) with a mean log2 ratio of approximately −1.0 for deletions and four adjacent oligos (probes) with a mean log2 ratio of approximately +0.6 for duplications. The clinical significance of CNVs was interpreted and reported in accordance with the current American College of Medical Genetics and Genomics standards and guidelines for interpretation and reporting of postnatal constitutional CNVs.[6] All reported abnormalities did not overlap with benign polymorphic CNVs. Databases used for interpretation of the clinical significance of CNVs included the Database of Genomic Variants, DECIPHER, ClinGen, OMIM, and PubMed. The UCSC Genome Browser was used to examine the gene content and to access all five databases. In this study a gene is considered a well-characterized haploinsufficient gene if shown in multiple peer-reviewed publications to result in a phenotype if involved by a heterozygous deletion or if it harbors a heterozygous loss-of-function mutation. Similarly, a gene is considered a well-characterized triplosensitive gene if shown in multiple peer-reviewed publications to result in a phenotype if involved by a duplication. On the other hand, a gene considered to have some evidence of dosage sensitivity is one described in a single case report to result in a phenotype if involved by a CNV and has a gene function that is relevant to the reason for patient referral. The ClinGen dosage sensitivity scores (https://www.ncbi.nlm.nih.gov/projects/dbvar/clingen/index.shtml) and/or the DECIPHER haploinsufficiency index scores were also included in the interpretation algorithm.[40]

Confirmatory studies included metaphase and interphase fluorescence in situ hybridization (FISH) testing using bacterial artificial chromosome clones from the RPCI-11 library (Empire Genomics, Buffalo, NY) for deletions between 100 and 500 kb in size and duplications between 400 and 500 kb. The clones' identities were confirmed by end sequencing and were directly labeled by nick translation using either SpectrumGreen or SpectrumOrange fluorescent dyes (Abbott, Abbott Park, IL). Deletions <100 kb and duplications <400 kb were confirmed by FISH using directly labeled oligonucleotide SureFISH probes (Agilent Technologies). All FISH probes were validated on normal metaphase chromosomes. CNVs with sizes smaller than the FISH resolution were confirmed by running the DNA sample on a higher-resolution aCGH platform. Samples originally run on the Agilent 4×44k aCGH or 8×60k aCGH arrays were rerun on the Agilent 2×105k aCGH array, whereas samples run on the Agilent 4×180k aCGH+SNP array were rerun on the same platform.

Data analysis

We examined small, nonrecurrent CNVs (<500 kb) among a cohort of 4,417 patients referred to our laboratory for CMA clinical testing. We examined these CNVs for their frequency, clinical significance, and phenotypic associations. The clinical significance of small pathogenic or likely pathogenic CNVs was reexamined during the course of this study. Also, we examined the gene content of the small pathogenic or likely pathogenic CNVs. Fisher's exact test was used to compare the proportions of the various phenotypic features between the groups of patients with small CNV (<500 kb) and the remaining patient cohort with abnormal aCGH results (**Table 4**).

RESULTS

In our laboratory we tested 4,417 patients using CMA technology over a period of 6 years, from 2009 through 2014. Of these patients, 383 (8.67%) were identified as carrying at least one small, nonrecurrent CNV (<500 kb). These 383 patients were identified to have a total of 435 small, nonrecurrent CNVs that were classified as pathogenic, likely pathogenic, or a variant of uncertain significance (VUS). We divided these patients into two groups: a pathogenic group and a VUS group. The pathogenic group consisted of patients with at least one small, nonrecurrent CNV classified as pathogenic or likely pathogenic and included 142 of the 4,417 patients (3.21%). This group included 113 patients (2.56%) with one small pathogenic or likely pathogenic CNV, 17 (0.38%) with two or more small pathogenic or likely pathogenic CNVs, and 12 (0.27%) with one small pathogenic or likely pathogenic CNV plus a recurrent CNV. The VUS group consisted of patients with one or more small, nonrecurrent VUS CNVs and included 241 of 4,417 patients (5.45%). This group included 176 patients (3.98%) with one small VUS CNV, 45 (1.02%) with one small VUS CNV plus other VUS CNVs, and 20 (0.45%) with one small VUS CNV plus a recurrent CNV. **Table 1** summarizes the patient breakdown by year for the pathogenic and VUS groups and the number/frequency of patients in each subcategory.

Of the 435 small, nonrecurrent CNVs, 147 were pathogenic or likely pathogenic and 288 were VUS CNVs. **Table 2** shows the abnormality type by year identified. Among the 147 pathogenic or likely pathogenic CNVs, 18 (12.2%) were homozygous or hemizygous deletions, 94 (63.9%) were heterozygous deletions, 34 (23.1%) were duplications, and 1 (0.7%) was a triplication. Reexamination of the clinical significance of CNVs in the pathogenic group resulted in reclassifying five of them from likely pathogenic to pathogenic as more evidence to support their pathogenicity was available in the literature. By contrast, the 288 VUS CNVs consisted of 14 (4.9%) hemizygous deletions, 148 (51.4%) heterozygous deletions, 112 (38.8%) duplications, and 14 (4.9%) triplications.

The patients' clinical presentation was thoroughly examined for all those with abnormal CMA results as part of our institutional review board–approved comprehensive genotype–phenotype database. However, we focused our analysis on the 142 patients identified in the pathogenic group. This included examining the gene content and genotype–phenotype correlations. A critical step in the interpretation of the clinical significance of small, nonrecurrent CNVs is thorough review of the patient's clinical presentation and its correlation with the available evidence in the literature and clinical CNV databases. This was possible for all patients in our cohort because all were referred within the same health system.

Within the pathogenic group, 80 of 142 (56%) patients were found to have a single whole-gene or exonic deletion that ranged in size from 9 to 440 kb. **Table 3** lists these genes, their functions, and the numbers of patients noted in our series with single-gene deletions. A total of 54 genes were noted to be targeted

Table 1 Distribution of patients with small, nonrecurrent CNVs in our series of 4,417 patients referred for CMA testing

Year	1 Small VUS CNV	1 Small VUS + ≥1 VUS CNVs	1 Small VUS + recurrent CNVs	At least 1 small pathogenic CNV	≥2 Small pathogenic CNVs	1 Small pathogenic + recurrent CNV	Patients with small CNVs
2009	9	2	2	10	2	1	26
2010	24	5	1	17	2	1	50
2011	38	6	4	24	2	1	75
2012	28	11	0	16	3	1	59
2013	40	11	5	24	3	4	87
2014	37	10	8	22	5	4	86
Total	176 (46.0%)	45 (11.7%)	20 (5.2%)	113 (29.5%)	17 (4.4%)	12 (3.1%)	383
Overall total	3.98%	1.02%	0.45%	2.56%	0.38%	0.27%	4,417[a]

CMA, chromosomal microarray; CNV, copy-number variant; VUS, variant of uncertain significance.

[a]Total number of patients tested in 2009–2014.

Table 2 Types of small, nonrecurrent CNVs in our series of 4,417 patients referred for CMA testing

	VUS CNVs (*n* = 288)				Pathogenic CNVs (*n* = 147)				
Year	Hemizygous deletion	Heterozygous deletion	Duplication	Triplication	Homozygous deletion	Hemizygous deletion	Heterozygous deletion	Duplication	Triplication
2009	0	1	17	0	0	1	12	2	0
2010	0	16	16	2	0	2	11	8	0
2011	0	32	20	3	1	4	19	2	1
2012	4	28	12	1	0	3	10	8	0
2013	6	29	28	6	0	1	27	3	0
2014	4	42	18	2	0	6	15	11	0
Total	14 (4.9%)	148 (51.4%)	112 (38.8%)	14 (4.9%)	18 (12.2%)		94 (63.9%)	34 (23.1%)	1 (0.7%)

CNV, copy-number variant; VUS, variant of uncertain significance.

Table 3 List of genes and their functions involved by single-gene deletions (<500 kb) in the pathogenic group

Gene	OMIM	Cyto band	Targeted on the array	Patients (*n*)	Gene function
CAMTA1	611501	1p36.31p36.23	–	2	Transcription activator
RERE	605226	1p36.23	–	1	Transcriptional corepressor
NFIA	600727	1p31.3	+	2	Transcription factor
COL11A1	120280	1p21.1	+	4[a]	Collagen chain component
NRXN1	600565	2p16.3	+	4	Neuronal adhesion molecule
EXOC6B	607880	2p13.2	–	2	Exocyst complex component
SATB2	608148	2q33.1	+	1	Transcriptional regulation and chromatin remodeling
AGAP1	608651	2q37.2	–	1	ARF-GAP protein
GRM7	604101	3p26.1	–	1	Glutamate neuronal receptor
MITF	156845	3p14.1p13	+	1	Transcription factor
CADM2	609938	3p12.1	–	1	Neuronal adhesion molecule
NLGN1	600568	3q26.31	–	1	Neuronal adhesion molecule
TP63	603273	3q28	+	2	Tumor suppressor
CTNND2	604275	5p15.2	–	1	Regulator of neuronal migration
CDH12	600562	5p14.3	–	1	Neuronal adhesion molecule
MEF2C	600662	5q14.3	–	2	Transcription factor
FOXC1	601090	6p25.3	+	1	Transcription factor
ARID1B	614556	6q25.3	–	1	Chromatin remodeling
GLI3	165240	7p14.1	+	2	Transcription factor
AUTS2	607270	7q11.22	+	3	Unknown function
GPR85	605188	7q31.1	–	1	G protein–coupled receptor
GATA4	600576	8p23.1	+	1	Transcription factor
PTCH1	601309	9p22.32	+	1	Tumor suppressor
EHMT1	607001	9q34.3	+	2	Histone methyltransferase
PAX6	607108	11p13	+	1	Transcription factor
DLG2	603583	11q14.1	–	2	Neuronal scaffold protein
MED13L	608771	12q24.21	–	1	Transcriptional coactivator
DACH1	603803	13q21.33	–	1	Transcription factor
CHD8	610528	14q11.2	+	1	Chromatin remodeling
NKX2-1	600635	14q13.3	–	2	Transcription factor
NRXN3	600567	14q24.3q31.1	–	2	Neuronal adhesion molecule
IGF1R	147370	15q26.3	+	1	Growth factor receptor
RNPS1	606447	16p13.3	–	2	RNA binding protein
CREBBP	600140	16p13.3	+	1	Transcriptional regulation and histone acetylation
CDH8	603008	16q21	–	1	Neuronal adhesion molecule
CTNS	606272	17p13.2	+	1[b]	Lysosomal transport
BRCA1	113705	17q21.31	–	1	Tumor suppressor
TCF4	602272	18q21.2	+	2	Transcription factor
DCC	120470	18q21.2	–	1	Netrin receptor
CDH19	603016	18q22.1	–	1	Neuronal adhesion molecule
GDF5	601146	20q11.22	+	1	Regulator of cell growth and differentiation
SHANK3	606230	22q13.33	+	1	Neuronal scaffold protein
SHOX	312865	Xp22.33/ Yp11.32	+	2[c]	Transcription factor
NLGN4X	300427	Xp22.32p22.31	+	1	Neuronal adhesion molecule
PTCHD1	300828	Xp22.11	–	1	Morphogen sonic hedgehog receptor
IL1RAPL1	300206	Xp21.3p21.2	+	2	Neurotransmission
DMD	300377	Xp21.2p21.1	+	4	Structural muscle protein
CYBB	300481	Xp11.4	+	1	Component of phagocytic NADPH-oxidase
CASK	300172	Xp11.4	+	2	Synaptic scaffold protein
OPHN1	300127	Xq12	+	1	Neuronal mediator of intracellular signal transduction
EDA2R	300276	Xq12	–	1	Transmembrane protein of the tumor necrosis factor receptor superfamily
CUL4B	300304	Xq24	+	1	Scaffold protein of the E3 ubiquitin ligase
SOX3	313430	Xq27.1	+	1	Transcription factor
MECP2	300005	Xq28	+	1[c]	Methylated DNA binding

With the exception of *SHOX* and *MECP2*, all chromosome X deletions are hemizygous deletions resulting in gene nullisomy. Underlined genes are well-characterized haploinsufficient genes.

[a]Intragenic multiexon deletions in *COL11A1* thought to result in in-frame transcripts. [b]A homozygous deletion in *CTNS*. [c]A heterozygous deletion in chromosome X.

by single-gene deletions, which included 24 well-characterized haploinsufficient genes, 16 genes with some evidence of dosage sensitivity, one gene with intragenic dominant-negative deletions, one recessive gene, and 12 X-linked OMIM morbid genes. As shown in **Table 3**, approximately half of these genes are targeted on our aCGH platforms. Examining the functions of these genes revealed that the majority of them are involved in transcriptional regulation (*CAMTA1*, *SATB2*, *MITF*, *MEF2C*, *FOXC1*, *GLI3*, *GATA4*, *PAX6*, *MED13L*, *NKX2-1*, *CREBBP*, *TCF4*, *SHOX*, *SOX3*), chromatin remodeling (*SATB2*, *ARID1B*, *CHD8*), histone covalent modification (*EHMT1*, *CREBBP*), methylated DNA binding (*MECP2*), neuronal axon guidance (*DCC*), neuronal synaptic function (*NRXN1*, *SHANK3*, *NLGN4X*, *CASK*, *OPHN1*), neurotransmission (*IL1RAPL1*), growth regulation (*IGF1R*, *GDF5*), cellular structure (*DMD*), and tumor suppression (*TP63*, *PTCH1*, *BRCA1*).

Among the 16 genes with some evidence of dosage sensitivity are several with growing evidence of haploinsufficiency, including *NFIA*, *CTNND2*, and *DLG2*. We present here five patients with deletions involving these three genes, which reinforces their pathogenicity. *NFIA* encodes a member of the nuclear factor I family of transcription factors that is critical for normal brain development and function (MIM 600727).[20,21] There is growing evidence that haploinsufficiency for *NFIA* is associated with a phenotype characterized by hypoplastic or absent corpus callosum, hydrocephalus, and developmental delay, and in some patients a tethered spinal cord, Chiari type I malformation, seizures, and urinary tract anomalies.[8,22–25] Two patients in our series had small deletions involving *NFIA*. Patient 99199 was noted to have a 254-kb deletion that encompasses exons 4 through 11 of *NFIA* (**Figure 1a**). We previously reported in detail this 25-year-old woman who presented with intellectual disability, bipolar disorder/depression, mild hydrocephalus, decreased volume of white matter, and hypoplasia of corpus callosum.[8] The patient has since moved to another state and is lost to follow-up. Patient 13857 was noted to have a 99-kb deletion that encompasses the last exon of *NFIA* (**Figure 1a**). This 1.5-year-old boy presented with global developmental delay (fine and gross motor and speech delays), prominent cavum septum pellucidum and cavum vergae, tethered spinal cord, right eye esotropia, mild ptosis, asymmetric movement of facial muscles, mild lower extremity spasticity, and cortical renal cysts. It is not clear at this point whether this deletion can trigger nonsense-mediated mRNA decay; however, based on the patient's phenotype it most likely results in haploinsufficiency without a dominant-negative effect. No additional genetic tests were ordered for this patient. His father, who presented with developmental delay and mild intellectual disability, carried the same deletion.

CTNND2 encodes the catenin delta 2 protein, which functions as a regulator of neuronal migration and maintenance of dendrites and dendritic spines in the mature cortex (MIM 604275).[14,15] It maps to the 5p region that is deleted in Cri du Chat syndrome and has been implicated in developmental delay, intellectual disability, autism, and schizophrenia.[7,16,17] Patient 13167 was noted to have a 145-kb deletion that encompasses exon 2 of *CTNND2* (**Figure 1b**) together with a recurrent 15q11.2 BP1-BP2 microduplication. This 13-month-old boy presented with central and obstructive sleep apnea, mild developmental delay, and partial complex seizures. He also presented with immune deficiency that is thought to be unrelated to his *CTNND2* deletion. His 4-year-old sister, who carries both abnormalities, presented with a similar phenotype, including mild developmental delay (gross motor and speech delays), seizures, and immune deficiency. In an attempt to find the cause of her immune deficiency, the proband's sister was studied by whole-exome sequencing, which was unrevealing; however, the proband was not tested by whole-exome sequencing. No phenotypic information was available on the parents, who declined parental FISH testing.

Another clinically significant gene with evidence of haploinsufficiency is *DLG2*, which encodes a member of the membrane-associated guanylate kinase family. The encoded protein forms a heterodimer with a related family member that interacts at postsynaptic sites to form a multimeric scaffold for clustering receptors, ion channels, and associated signaling proteins (MIM 603583). Recent reports in the literature suggest that haploinsufficiency for *DLG2* is associated with intellectual disability, autism, seizures, and psychiatric disorders.[18,19] Two patients in our series had small deletions involving *DLG2*. A 271-kb deletion that encompasses exons 3 through 5 of *DLG2* was noted in patient 13215 (**Figure 1c**), together with a recurrent *STS* gene (Xp22.31) microduplication. This 8-month-old boy presented with intrauterine growth retardation, mild dysmorphic features, and bilateral cleft lip and palate. He was reexamined at 2 years of age and was showing signs of learning disability. Both parents were reported as being normal and in good general health, but they declined parental FISH testing. The second patient is a 4-year-old girl (patient 13103) who presented with developmental delay, little speech, autism spectrum disorder, and dysmorphic features. She was noted to have a 102-kb deletion that encompasses the first exon of the shortest splice form of *DLG2* (**Figure 1c**). The mother received a clinical diagnosis of Stickler syndrome as a child, and the father reportedly did not talk until age 4 and had a hard time in school. Both parents were not available for testing. No additional genetic tests were ordered for both patients with deletions involving the *DLG2* gene.

Four patients presenting with clinical features suggestive of Stickler syndrome type 2 had intragenic multiexon deletions in the *COL11A1* gene that are thought to result in in-frame transcripts with a dominant-negative effect (MIM 604841).[39] One patient presenting with nephropathic cystinosis had a 15-kb homozygous deletion in the *CTNS* gene (MIM 219800).

Phenotypic features were collected for all patients with abnormal aCGH results. **Table 4** provides a detailed list of all types of abnormalities noted in our patient cohort. The phenotypic checklist including details of the types of abnormalities included in each category is available in **Supplementary**

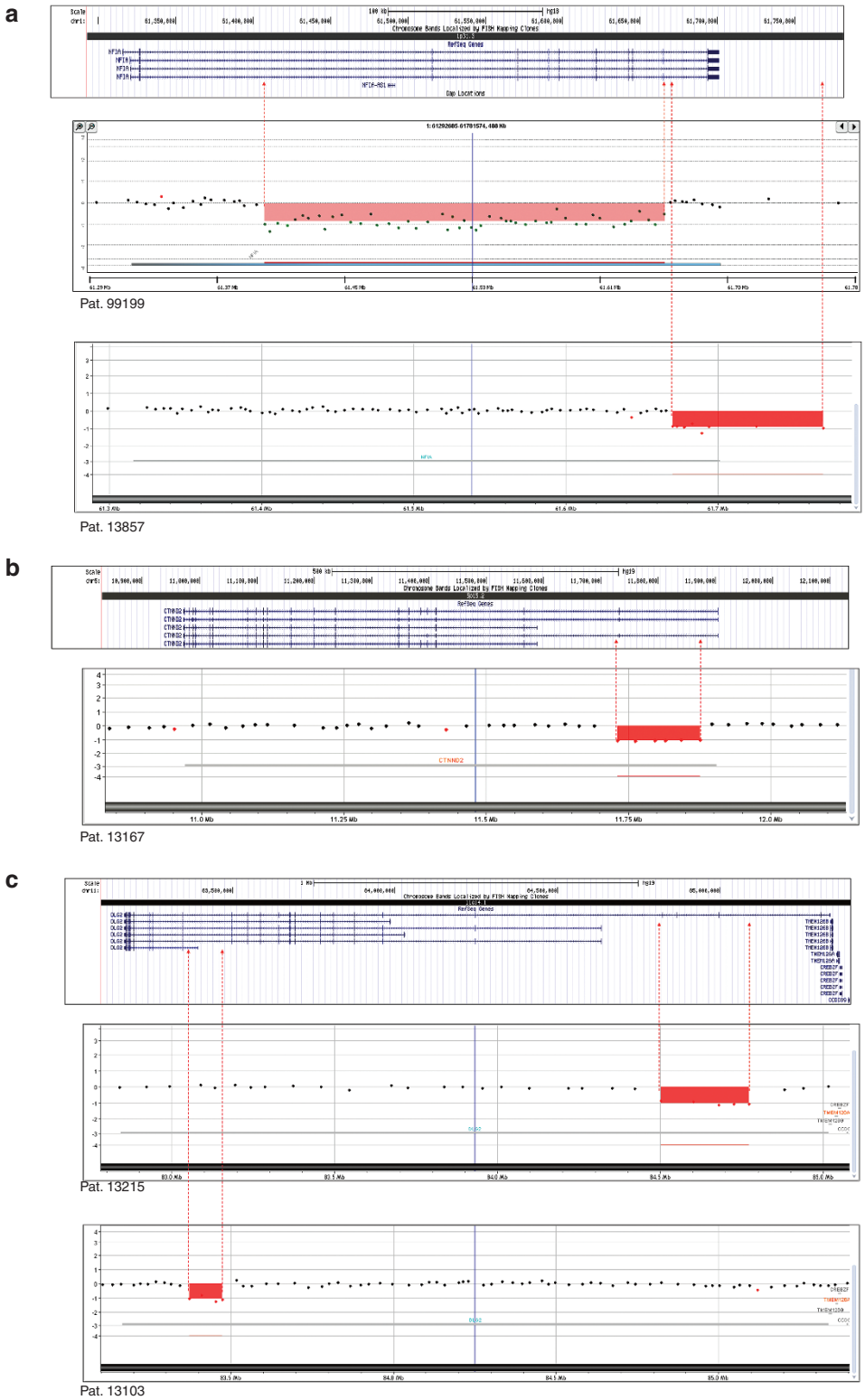

Figure 1 Oligo array comparative genomic hybridization (aCGH) plots aligned with a genomic map of the same region generated using the UCSC Genome Browser and showing the deleted region (shaded area and dashed lines), genomic ruler, cytogenetic band, and annotated RefSeq gene. (**a**) Patient 99199, run on the Agilent 2×105k aCGH array, demonstrating a 254-kb deletion that encompasses exons 4 through 11 of *NFIA*. Patient 13857, run on the Agilent 8×60k aCGH array, demonstrating a 99-kb deletion that encompasses the last exon of *NFIA*. (**b**) Patient 13167, run on the Agilent 4×180k aCGH+SNP array, demonstrating a 145-kb deletion that encompasses exon 2 of *CTNND2*. (**c**) Patient 13215, run on the Agilent 8×60k aCGH array, demonstrating a 271-kb deletion that encompasses exons 3 through 5 of *DLG2*. Patient 13103, run on the Agilent 4×180k aCGH+SNP array, demonstrating a 102-kb deletion that encompasses the first exon of the shortest splice form of *DLG2*.

Table 4 Phenotypic features of our patient series

Type of anomaly	Patients with pathogenic or likely pathogenic CNVs <500 kb (*n* = 142)	*P* value[a]	Patients with VUS CNVs <500 kb (*n* = 241 patients)	*P* value[b]	Remaining patients with abnormal aCGH results (*n* = 862 patients)
Growth	34 (23.9)	1	53 (22)	0.49	209 (24.2)
Cognitive and developmental disabilities	104 (73.2)	0.01*	142 (58.9)	0.37	536 (62.2)
Behavioral and psychiatric	27 (19)	0.91	54 (22.4)	0.42	171 (19.8)
Neurological	60 (42.3)	0.71	106 (44)	0.30	347 (40.3)
Craniofacial	99 (69.7)	0.12	168 (69.7)	0.055	654 (75.9)
Craniofacial—dysmorphic features only	53 (37.3)	0.41	86 (35.7)	0.12	356 (41.3)
Cardiac	17 (12)	0.035*	36 (14.9)	0.13	167 (19.4)
Musculoskeletal	33 (23.2)	0.004*	80 (33.2)	0.54	306 (35.5)
Gastrointestinal	8 (5.63)	0.12	23 (9.5)	0.9	87 (10.1)
Genitourinary	16 (11.3)	0.04*	38 (15.8)	0.39	158 (18.3)
Hearing and vision	23 (16.2)	0.1	48 (19.9)	0.43	194 (22.5)
Cutaneous	5 (3.5)	0.0004*	30 (12.4)	0.83	115 (13.3)

Data are *n* (%) unless otherwise indicated.

[a]*P* value compares the proportions of the various phenotypic features between the small pathogenic copy-number variants (CNVs; <500 kb) patient group and the remaining patient cohort with abnormal array comparative genomic hybridization (aCGH) results. [b]*P* value compares the proportions of the various phenotypic features between the small variant of uncertain significance CNV (<500 kb) patient group and the remaining patient cohort with abnormal aCGH results. *Statistically significant difference ($P \leq 0.05$).

Table S1 online. Within the small pathogenic CNV group (142 patients), cognitive and developmental abnormalities were the most common features (73.2%), encompassing all types of developmental delay (gross motor, fine motor, or speech), intellectual disability, or learning disability. Behavioral and psychiatric abnormalities such as autism spectrum disorders, attention deficit hyperactivity disorder, and general anxiety disorder were reported in 19% of patients in the same group, whereas other neurological conditions, such as seizures, structural brain abnormalities, and abnormal muscle tone, were reported in 42.3% of patients. Approximately 69.7% of the patients within the small pathogenic CNV group presented with craniofacial anomalies such as cleft lip with or without cleft palate, microcephaly, macrocephaly, and dysmorphic features. Many patients presented with more than one type of abnormality. Comparison of the proportions of the various phenotypic features between the small pathogenic CNV group and the remaining patient cohort with abnormal aCGH results demonstrated a significant excess of cognitive and developmental disabilities in the small pathogenic CNV group ($P = 0.01$), whereas the same group demonstrated significantly smaller proportions of cardiac, musculoskeletal, genitourinary, and cutaneous anomalies ($P = 0.035$, 0.004, 0.04, and 0.0004, respectively). On the other hand, the small VUS CNV group did not demonstrate significant differences in the proportions of the various phenotypic features when compared with the remaining patient cohort with abnormal aCGH results (**Table 4**).

DISCUSSION

High-resolution CMA technology has significantly improved the resolution of cytogenetic analysis in detecting small genomic losses and gains that are relevant to human genetic disease. Despite extensive efforts to understand the clinical significance of CNVs and standardize their reporting criteria, variability among laboratories remains.[1,2,4–6,10] Small, nonrecurrent CNVs (<500 kb) present a significant challenge to clinical laboratories. As part of our ongoing effort to assemble a comprehensive genotype–phenotype database for our patients with abnormal CMA results, this study was designed to focus on all nonpolymorphic small, nonrecurrent CNVs (<500 kb) in patients referred to our laboratory for CMA clinical testing over a period of 6 years, from 2009 to 2014 (a total of 4,417 patients). We examined their frequency, clinical significance, gene content, and phenotypic associations.

Our study showed that 8.67% of all the patients tested had at least one small, nonrecurrent CNV. These patients had a total of 435 small, nonrecurrent CNVs. Approximately one-third of these CNVs were classified as pathogenic or likely pathogenic (**Table 2**) and were observed in 3.21% of the total number of patients tested (**Table 1**). This represents a significant added value of small, nonrecurrent pathogenic or likely pathogenic CNVs (<500 kb) to the diagnostic yield of CMA clinical testing. Our findings of ~3% of our patients with pathogenic or likely pathogenic CNVs <500 kb is slightly higher than the reported 0.4–2% observed in previous studies.[7,8,26] This could be explained by the larger number of targeted genes on our aCGH platforms, as shown in **Table 3**, as well as the interpretation of CNV clinical significance in the context of the patients' detailed clinical information, which allows better genotype–phenotype correlations with the available evidence for pathogenicity.

Approximately 56% (80/142) of the patients within the pathogenic group had a small deletion <500 kb involving a single clinically relevant autosomal or X-linked gene. A total of 54 genes were noted to be targeted by these small deletions (**Table 3**), which includes 24 well-characterized haploinsufficient genes, 16 genes with some evidence of dosage sensitivity, 1 gene with intragenic dominant-negative deletions, 1 recessive gene, and 12 X-linked OMIM morbid genes. A closer examination of the

functions of these genes revealed that the majority of them are involved in transcriptional regulation, chromatin remodeling, histone covalent modification, neuronal axon guidance, neuronal synaptic function, neurotransmission, growth regulation, and tumor suppression. Our results are probably skewed toward genes that function within neurodevelopmental pathways because of the higher number of these genes targeted on our aCGH platforms. Nineteen genes were noted to be targeted by single-gene deletions in more than one patient, which suggests that they are frequent targets for small deletions (**Table 3**).

Among the 16 genes involved by single-gene deletions and with some evidence of dosage sensitivity, we noted three genes (*NFIA*, *CTNND2*, and *DLG2*) with growing evidence for haploinsufficiency. We and others have described the novel syndrome associated with *NFIA* haploinsufficiency,[8,22–25] but haploinsufficiency for *CTNND2* and *DLG2* has been reported in only a few cases.[7,16–19] Our findings reinforce the phenotype of developmental delay, intellectual disability, and autistic features associated with *CTNND2* and *DLG2* haploinsufficiency. No exonic deletions in any of the three genes have been reported in the Database of Genomic Variants. Also, no frameshift or nonsense mutations have been reported in these genes in the normal population, as shown in the HapMap and the 1000 Genomes Project databases. Other clinically relevant genes on this list with some evidence of haploinsufficiency include *RERE* (1p36.23),[28] *EXOC6B* (2p13.2),[29,30] *AGAP1* (2q37.2),[31] *GRM7* (3p26.1),[32] *NLGN1* (3q26.31),[33] *CDH12* (5p14.3),[34] *GPR85* (7q31.1),[35] *NRXN3* (14q24.3q31.1),[36] *RNPS1* (16p13.3),[37] and *CDH8* (16q21).[34,38] The three remaining genes (*CADM2*, *DACH1*, and *CDH19*) are potentially clinically relevant based on their function and/or DECIPHER haploinsufficiency index score.[40] Further investigation of the clinical significance of deletions/duplications involving these genes is required. Similar future studies can add the power required to identify additional novel genes involved in the pathogenesis of neurodevelopmental disorders and/or congenital anomalies. This will require better data sharing, particularly among academic clinical laboratories with full access to patients' clinical information.[6,7] It can be achieved by depositing abnormal CMA results along with well-characterized phenotypes into publicly available clinical CNV databases.[12,13] This strategy can be extended to include small, nonrecurrent VUS CNVs that involve single genes; however, we did not research these genes in full detail in this study. Our laboratory has been actively participating in sharing abnormal CMA results through ClinVar/ClinGen, which includes all retrospective data since January 2009.

As shown in **Table 1**, 4.4% of patients with small, nonrecurrent CNVs were found to have two or more small pathogenic or likely pathogenic CNVs, which did not include recurrent microdeletions and microduplications. Likewise, 11.7% of patients were found to have one small VUS CNV plus one or more other VUS CNVs. Also, 8.3% of patients with either small, nonrecurrent pathogenic or VUS CNVs were identified to have a small recurrent CNV. These included the 15q11.2 BP1-BP2 CNVs, *CHRNA7* (15q13.3) duplication, proximal 16p11.2 CNVs, distal 16p11.2 deletion, and *STS* (Xp22.1) duplication in males. Collectively, ~24% of patients with small, nonrecurrent CNVs (2.12% of the total patient cohort) carried more than one CNV, which indicates that the increased CNV burden in these patients could lead to a more severe phenotype, as discussed in the two-hit hypothesis suggested by Girirajan et al.[11]

The frequency of small, nonrecurrent deletions (homozygous, hemizygous, and heterozygous) within the pathogenic group was three times that of duplications (**Table 2**). These duplications either span genes with some evidence of triplosensitivity or are intragenic duplications that can lead to out-of-frame defects and gene haploinsufficiency. This higher frequency of small pathogenic deletions was noted in previous studies using either high-resolution CMAs or whole-exome sequencing.[7,9,27]

Small CNVs <500 kb have previously been reported in individuals with a variety of neurodevelopmental phenotypes.[7–9] Our genotype—phenotype correlations clearly demonstrated that the predominant phenotypes in patients with small pathogenic or likely pathogenic CNVs were developmental delay and/or intellectual disability (73.2%), which were significantly more common in this CNV group when compared with the remaining patient cohort with abnormal aCGH results. This probably reflects a positive correlation with the large proportion of small pathogenic deletions involving neurodevelopmental genes observed in this group (**Table 3**). On the other hand, the small pathogenic CNV group demonstrated significantly smaller proportions of cardiac, musculoskeletal, genitourinary, and cutaneous anomalies when compared with the remaining patient cohort with abnormal aCGH results (**Table 4**).

The main strength of this study is the amount of clinical information available for each patient. The majority of our patients were examined at the University of Alabama at Birmingham General Genetics Clinic or the affiliated Children's Hospital of Alabama. Therefore, the laboratory directors and laboratory genetic counselor had access to the patients' electronic medical records. This level of clinical information is rarely available to clinical laboratories, limiting the interpretation of the clinical significance of CMA abnormalities. This highlights the importance of providing complete and thorough phenotypic information for all genetic testing laboratories. Without this information, laboratories cannot accurately interpret the clinical significance of rare CNVs.

One limitation of this study was the inability to perform testing in the parents of many patients. Possible explanations include the relatively high frequency of adopted children, a decline in parental testing, and/or lack of insurance coverage.

Several themes emerged from our study. First, ~3% of our total patient cohort carried at least one small pathogenic or likely pathogenic CNV. Second, ~56% of patients within the small pathogenic CNV group carried a single whole-gene or exonic deletion involving a clinically relevant autosomal or X-linked gene. Third, ~24% of patients with small, nonrecurrent CNVs (2.12% of the total patient cohort) carried more than one CNV.

In conclusion, our study demonstrates the diagnostic clinical relevance of small (<500 kb) nonrecurrent CNVs during CMA

clinical testing and underscores the need for careful clinical interpretation of these CNVs. These small, nonrecurrent CNVs can also facilitate the discovery of new genes involved in the pathogenesis of neurodevelopmental disorders and/or congenital anomalies.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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