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Two Patients with Complex Rearrangements Suggestive of Germline Chromoanagenesis

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Established Facts

- Rare constitutional chromoanagenesis events have been reported in a limited number of patients with variable phenotypes.
- A preferential bias for paternal origin has been described in germline chromothripsis-mediated events, but no parental bias has so far been described for chromoanasynthesis-derived rearrangements.

Novel Insights

- We add 2 additional cases suggestive of constitutional chromoanagenesis to the literature, with our first case representing the oldest known patient.
- Our cases may add evidence to support a paternal bias in constitutional chromoanasynthesis events, similar to what has been observed in cases of germline chromothripsis.

Keywords
Germline chromoanagenesis · Chromoanasynthesis · Chromothripsis · Complex chromosomal rearrangement · Constitutional events

Abstract
Chromoanagenesis, a phenomenon characterized by complex chromosomal rearrangement and reorganization events localized to a limited number of genomic regions, includes the subcategories chromothripsis, chromoanasynthesis, and chromoplexy. Although definitions of these terms are evolving, constitutional chromoanagenesis events have been reported in a limited number of patients with variable phenotypes. We report on 2 cases with complex genomic events characterized by multiple copy number gains and losses confined to a single chromosome region, which are suggestive of constitutional chromoanagenesis. Case 1 is a 43-year-old male with intellectual disability and recently developed generalized tonic-clonic seizures. Chromosomal microarray analysis identified a complex rearrangement involving chromosome region 14q31.1q32.2, consisting of 16

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breakpoints ranging in size from 0.2 to 6.2 Mb, with 5 segments of normal copy number present between these alterations. Interestingly, this case represents the oldest known patient with a complex rearrangement indicative of constitutional chromoanagenesis. Case 2 is a 2-year-old female with developmental delay, speech delay, low muscle tone, and seizures. Chromosomal microarray analysis identified a complex rearrangement consisting of 28 breakpoints localized to 18q21.32q23. The size of the copy number alterations ranged from 0.042 to 5.1 Mb, flanked by 12 small segments of normal copy number. These cases add to a growing body of literature demonstrating complex chromosomal rearrangements as a disease mechanism for congenital anomalies.

Introduction

Complex chromosomal rearrangements leading to localized structural reorganization of genomic DNA are catastrophic cellular events represented collectively by the term "chromoanagenesis" [Kloosterman et al., 2011; Liu et al., 2011; Stephens et al., 2011; Holland and Cleveland, 2012; Masset et al., 2016]. Analysis of breakpoint junction sequences and investigation of the underlying mechanisms associated with chromoanagenesis has led to the recognition of 3 main categories: chromothripsis, chromoplexy, and chromoanasynthesis [Liu et al., 2011; Stephens et al., 2011; Holland and Cleveland, 2012; Baca et al., 2013; Fukami et al., 2017; Ly and Cleveland, 2017; Pellestor, 2019; Zepeda-Mendoza and Morton, 2019]. Chromothripsis refers to a phenomenon of localized chromosomal shattering and random reorganization of chromosome segments, typically restricted to a single chromosome, and is often associated with deletions [Stephens et al., 2011]. In chromoplexy, derivative chromosomes are generated by a series of chained rearrangements involving segments of DNA from multiple chromosomes that maintain a largely balanced DNA content [Baca et al., 2013]. In contrast, chromoanasynthesis is characterized by alternating copy number changes including a combination of deletions, duplications, and triplications arising from defective replication, typically clustered on a single or a few chromosomes [Liu et al., 2011].

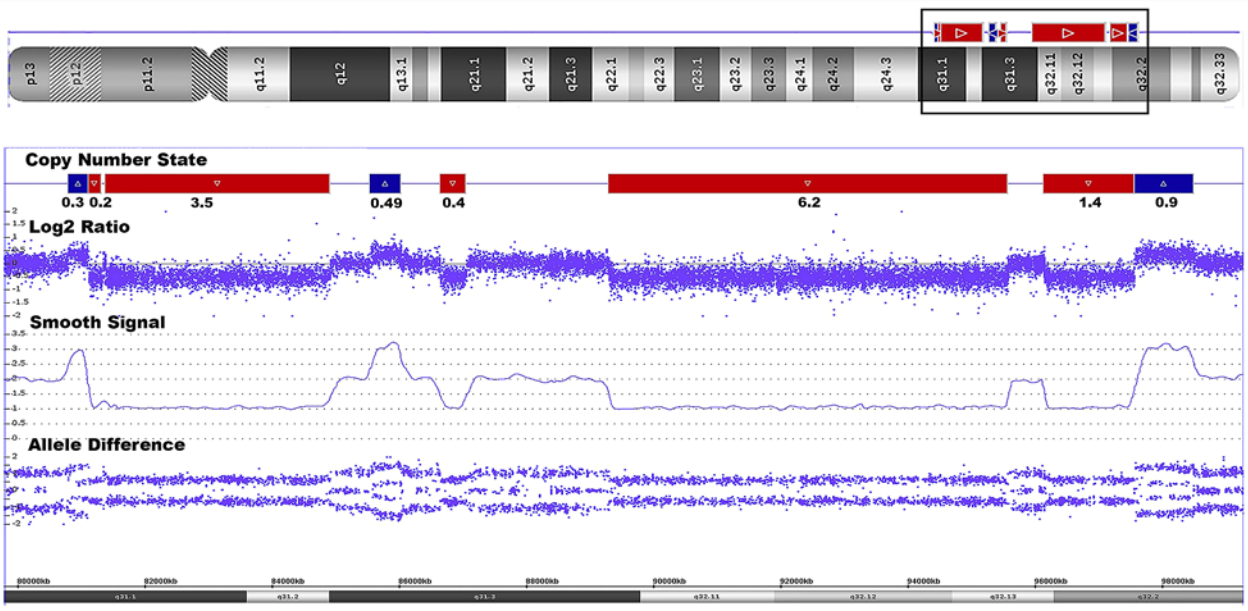
In contrast to chromothripsis and chromoplexy, which have been documented in both cancer and rare constitutional cases, chromoanasynthesis has been primarily associated with complex rearrangements in the germline. Collectively, germline chromoanagenesis-compatible structural rearrangements have been reported in patients with variable neurodevelopmental phenotypes and apparently normal individuals ascertained through an affected family member [Piccione et al., 2010; Kloosterman et al., 2011, 2012; Liu et al., 2011; Genesio et al., 2013, 2015; Gu et al., 2013; Kloosterman and Cuppen, 2013; Fontana et al., 2014; Nazaryan et al., 2014; Plaisancié et al., 2014; de Pagter et al., 2015; Gamba et al., 2015; Wang et al., 2015; Weckselblatt et al., 2015; Anderson et al., 2016; Bertelsen et al., 2016; Burnside et al., 2016; Del Rey et al., 2016; Masset et al., 2016; Fukami et al., 2017; Sabatini et al., 2018; Gudipati et al., 2019; Koltsova et al., 2019; Nazaryan-Petersen et al., 2019; Zepeda-Mendoza and Morton, 2019; Ader et al., 2020]. An additional 2 cases have been identified prenatally [Macera et al., 2015; Bone et al., 2019]. Published cases include inherited chromoanagenesis events, either balanced or unbalanced, as well as those that have arisen de novo during gametogenesis or early embryogenesis. Studies that have identified the parental origin of these events suggest a bias toward paternal alleles in chromothripsis-mediated rearrangements, while no parental bias has been established for chromoanasynthesis events [Kloosterman et al., 2011, 2012; Liu et al., 2011; Weckselblatt and Rudd, 2015; Weckselblatt et al., 2015; Collins et al., 2017; Fukami et al., 2017; Zepeda-Mendoza and Morton, 2019]. Given the overall rarity of chromoanagenesis in the germline, the now-routine clinical use of high-resolution chromosomal microarray analysis (CMA) presents an increased opportunity to uncover these complex events that would have gone largely undetected by conventional cytogenetics, and allows for further exploration of their mechanism and significance in congenital disease.

In this study, we describe localized complex rearrangements in 2 patients evaluated by CMA in our clinical diagnostic laboratory. The genomic rearrangements, each confined to a single chromosome arm, produced an oscillating copy number pattern with segments of disomy present between most of the gains and losses, resembling the characteristic features of germline chromoanagenesis. Additionally, we determined the parental origin of the rearranged chromosomes.

Materials and Methods

Chromosome Analysis

Peripheral blood specimens were cultured, harvested, and prepared for GTG-banding (G-banding using trypsin and Giemsa) using standard cytogenetic methods. G-banded metaphase chromosomes were analyzed and captured at or above the 500-band level.



a

b

Chromosme 14 Coordinate	SNP Marker	Paternal Genotype	Maternal Genotype	Proband's Genotype
80855181	S-3SPZD	AB	AA	ABB
81047974	S-4IPYJ	AB	AA	ABB
81144044	S-4LDEF	AA	BB	B
81144142	S-3ERHP	AA	BB	B
81144750	S-3YQWL	AA	BB	B
85698743	S-3ARWP	BB	AA	ABB
85906139	S-4FJUN	AA	BB	AAB
85913426	S-3TJCB	BB	AA	ABB
86706476	S-4DUVA	AA	BB	B
86708333	S-4BBDM	BB	AA	A
86709107	S-4IOCK	AA	BB	B
98123005	S-3IUDS	AB	BB	AAB
98177573	S-3KEKA	AB	AA	ABB
98408648	S-3BOIU	AA	BB	AAB
98409403	S-4NJSI	BB	AA	ABB

c

Fig. 1. Complex chromosomal rearrangement involving chromosome 14 in case 1. **a** The boxed area on the chromosome 14 idiogram indicates the region of detail shown below it. Chromosomal microarray data depict the complex chromosomal rearrangement confined to 14q31.1q32.2 (80,785,657–98,490,569 [hg19]). The alternating copy number losses and gains are indicated by the red and blue bars, respectively, with the size of individual CNVs listed below each in Mb. Copy number data (Log2 ratio, smooth signal) and genotype information (allele difference) are represented as individual tracks on the array plot. **b** G-banded partial karyotype showing the interstitial deletion in one chromosome 14 (arrow). **c** Genotyping data for the trio are represented by selected informative SNPs with specified chromosomal position and corresponding SNP marker. The paternal genotype is shown in red, the maternal genotype in black, and the derived genotype for the proband is depicted for duplications and deletions along the length of chromosome 14.

Table 1. Specific intervals, size of copy number variants, and genes in the 14q region for case 1

Genomic region [hg19]	Size, Mb	RefSeq genes in the region	Classification
14q31.1(80792017_81108735)×3	0.32	*DIO2-AS1, CEP128*	VUS
14q31.1(81113098_81315110)×1	0.2	*CEP128*	VUS
14q31.1q31.3(81378210_84909793)×1	3.5	*CEP128, TSHR, GTF2A1, SNORA79, LOC101928504, STON2, LOC100506700, SEL1L, LINC01467*	VUS
14q31.3(85544758_86033063)×3	0.57	*LOC105370605, LINC00911, FLRT2*	VUS
14q31.3(86647802_87050689)×1	0.4	No genes	Likely benign
14q31.3q32.13(89297735_95572111)×1	6.2	*TTC8, FOXN3, FOXN3-AS1, FOXN3-AS2, EFCAB11, TDP1, KCNK13, PSMC1, NRDE2, CALM1, LINC00642, LOC105370619, TTC7B, LOC101928909, LOC105370622, RPS6KA5, C14orf159, SNORA11B, GPR68, CCDC88C, PPP4R3A, CATSPERB, TC2N, FBLN5, TRIP11, ATXN3, NDUFB1, CPSF2, SLC24A4, RIN3, LGMN, GOLGA5, LOC101929002, CHGA, ITPK1, ITPK1-AS1, MOAP1, TMEM251, C14orf142, UBR7, BTBD7, UNC79, COX8C, PRIMA1, FAM181A-AS1, FAM181A, ASB2, MIR4506, LINC00521, OTUB2, DDX24, IFI27L1, IFI27, IFI27L2, PPP4R4, SERPINA10, SERPINA6, SERPINA2, SERPINA1, SERPINA11, SERPINA9, SERPINA12, SERPINA4, SERPINA5, SERPINA3, SERPINA13P, GSC, DICER1*	Pathogenic
14q32.13q32.2(96143024_97560950)×1	1.4	*TCL1B, TCL1A, TUNAR, C14orf132, BDKRB2, BDKRB1, ATG2B, GSKIP, AK7, LOC730202, PAPOLA, VRK1, LINC00618*	VUS
14q32.2(97561323_98492843)×3	0.93	*LOC101929241, LOC100129345, LINC01550*	VUS

VUS, variant of uncertain significance.

Chromosomal Microarray Analysis

Genomic DNA was extracted from whole blood using the EZ1 Advanced XL automated DNA extraction method (Qiagen, Hilden, Germany) or from buccal swab samples (ORAcollect Dx kit) using the PrepIT L2P DNA extraction kit (DNA Genotek, Ottawa, ON, Canada). CMA was performed on the patients' DNA using the Applied Biosystems CytoScan HD array (ThermoFisher Scientific, Carlsbad, CA) consisting of approximately 1.9 million copy number and 750,000 SNP oligonucleotide probes. Data analysis was performed using Chromosome Analysis Suite (ChAS) version 3.3.0.139, with the following filtering criteria: deletions ≥25 kb (minimum 25 probes) and duplications ≥50 kb (minimum 50 probes). The results were analyzed and reported using the NCBI human genome build 37.1 (GRCh37/hg19).

SNP Genotype Analysis

SNP genotype calls were obtained from the ChAS software for the patients and their parents. Trio analysis for Case 1 and duo analysis for Case 2 were performed along the length of the copy number alterations on chromosomes 14 and 18, respectively. For regions of deletion, the SNP genotype was hemizygous in the proband and could be directly compared to the parental genotype. For regions of duplication, SNP genotypes were derived by analyzing allele differences corresponding to AAA = 1.5, AAB = 0.5, BBA = −0.5, BBB = −1.5. Parent of origin for the rearranged chromosome was determined by comparing the informative SNP calls for the patients and their parents in regions of chromosomal deletion and/or duplication.

Case Reports and Results

Case 1

The patient is a 43-year-old Caucasian male with an extensive medical history that includes developmental delay, intellectual disability, microcephaly, progressive muscle weakness, hypotonia, and late-onset seizures. At birth, he had respiratory distress and was reported to have spina bifida occulta. The patient walked at age 4 and was noted to have speech, cognition, and motor delays. He received special education in school until he was 18 years old. The patient is non-verbal but creates signals and signs to communicate with his mother. He can assist with dressing and feeding himself but otherwise depends on his mother's support. At age 18, G-banded chromosome analysis was performed by an outside laboratory which demonstrated a chromosome 14 deletion. At age 40, the patient developed generalized tonic-clonic seizures, and CMA was requested as part of a neurological workup. His seizures have been managed well with the use of Levetiracetam (Keppra). A recent clinical genetics evaluation identified minor malformations including prominent ears with underdeveloped helices and Darwin tubercles,

convexity of the nose with a hypoplastic malar region, deep-set eyes with periorbital purple discoloration, and tapered, long thin fingers with slight clubbing. The patient was noted to have markedly reduced muscle tone globally. The family history was notable for maternal recurrent miscarriages.

CMA detected a complex chromosomal rearrangement confined to 14q31.1q32.2 (coordinates 80,785,657–98,492,843 [hg19]) with 16 breakpoints, including 5 losses and 3 gains for a total of approximately 11.8 and 1.82 Mb of genomic material, respectively. The size of the copy number variants ranged from 0.2 to 6.2 Mb with 5 segments of normal copy number present between these alterations (Fig. 1a). The genomic coordinates and genes in the affected interval are summarized in Table 1. Most of the copy number changes are of uncertain clinical significance; however, a copy loss of 6.2 Mb at 14q31.3q32.13 was classified as pathogenic (Table 1). This deletion includes 44 OMIM-annotated genes, and similar deletions have been reported in patients with microcephaly, dysmorphic facial features, intellectual disability, and developmental delay [Piccione et al., 2010]. Conventional G-banding was performed in conjunction with the CMA study, and results suggested a simple deletion on the distal long arm of chromosome 14 at band 14q32.1, with no evidence of a complex rearrangement (Fig. 1b). The patient's parents were both negative for these copy number rearrangements by CMA (data not shown) indicating this was a de novo event; however, a balanced event in the parents cannot be completely excluded since chromosome analysis was not performed. The parental origin of the abnormal chromosome 14 was determined by SNP genotyping as described in the methods section. In the deleted intervals of 14q31.3q32.13 in the proband, only maternal alleles were observed, indicating loss of paternal alleles and paternal derivation of the abnormal chromosome 14. Further, the informative SNP markers within the duplicated interval of chromosome 14 were consistent with the additional 14q segments originating from the paternally derived chromosome (Fig. 1c).

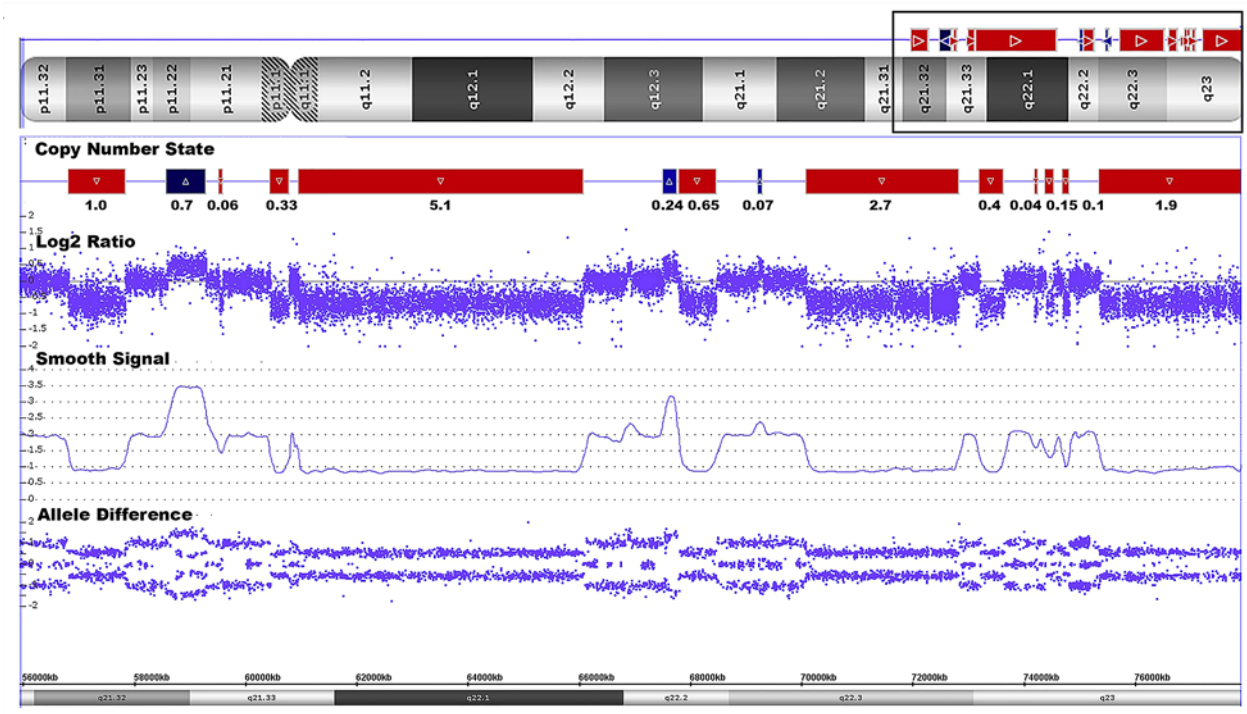
Case 2

The patient is a 2-year-old female with a history of seizures, developmental delay, poor growth, and hypotonia. There is limited pregnancy/birth history available as the child is in the care of a foster family. Following her birth, the patient had neonatal abstinence syndrome and was reportedly positive for 5 different substances. At 16 months of age, she developed seizures documented by an abnormal EEG, characterized with focal slowing, maximal over the right centroparietal-occipital region, and at times with a sharp configuration. Her seizures are currently under control with the use of Levetiracetam (Keppra). A head CT scan showed an open fontanelle and abnormal metopic suture, while her head MRI was normal. Due to the patient's history of developmental delay and seizures, CMA was requested, and the patient was referred for genetic evaluation following these results. The clinical genetics evaluation at 22 months of age documented mild dysmorphic features including an underdeveloped philtrum, thin vermillion border (both appoximately 4 on Likert scale), and bilaterally low-set ears with normal architecture. The patient has a wide-based gait and some imbalance, she wears corrective lenses for hyperopia and is noted to have delayed tooth eruption and frequent ear infections. The patient babbles frequently and can speak 25–30 words. She is currently enrolled for physical, occupational, and developmental therapies through First Steps.

CMA identified a complex genomic rearrangement consisting of 28 breakpoints at 18q21.32q23 (56,820,380–77,916,234 [hg19]) with 11 losses and 3 gains including a triplication for a total of 13.2 and 1.0 Mb of genomic material affected, respectively. The size of the copy number alterations ranged from 0.042 to 5.1 Mb with 12 small segments of normal copy number flanking these intervals (Fig. 2a). The specific intervals and genes within each of the copy number changes are summarized in Table 2. The concurrent G-banded chromosome analysis identified an apparently terminal deletion of chromosome 18q (Fig. 2b). To ascertain the parent of origin, duo analysis was performed as the mother's sample could not be obtained. The biological father was determined to be negative for complex rearrangements on chromosome 18 (data not shown). SNP genotyping showed the presence of only maternal (non-paternal) alleles in the deleted regions of chromosome 18q21.32q23, suggesting that these complex rearrangements had arisen on the paternal chromosome (Fig. 2c).

Discussion and Conclusion

Here, we describe 2 new cases of complex intrachromosomal rearrangements with CMA profiles suggestive of germline chromoanagenesis events. While rare, chromoanagenesis in the constitutional setting has been reported in increasing frequency since the first description of the phenomenon in cancer, and is likely an underappreciated phenomenon in the germline [Piccione et al.,



a

b

Chromosome 18 Coordinate	SNP Marker	Paternal Genotype	Proband's Genotype
56827121	S-3LTKN	BB	A
56827485	S-4IVMR	BB	A
56832307	S-4RNAV	AA	B
56877537	S-4JHDY	AA	B
56878051	S-4HJEX	AA	B
56878511	S-3XEZA	AA	B
56878948	S-3VFOB	AA	B
56913967	S-3TPFK	BB	A
56920558	S-3ZGRN	AA	B
56921310	S-4MXNO	BB	A
68062692	S-3BXRR	BB	A
68062834	S-4PYMZ	BB	A
73481134	S-3NXKH	AA	B
73481577	S-4FZQD	AA	B
73486030	S-4FIBG	AA	B

c

Fig. 2. Complex chromosomal rearrangement involving chromosome 18 in case 2. **a** The boxed area on the chromosome 18 idiogram illustrates the region of detail shown below it. Chromosomal microarray data depict the complex chromosomal rearrangement at 18q21.32q23 (56,820,380–77,916,234 [hg19]). The alternating copy number losses and gains are indicated by the red and blue bars, respectively, with the size of individual CNVs listed below each in Mb. Copy number analysis (Log2 ratio, smooth signal) and genotype information (allele difference) are represented as individual tracks on the array plot. **b** G-banded partial karyotype showing the terminal deletion in one chromosome 18 (arrow). **c** Genotyping data for the duo (father and child) are represented for selected informative SNPs with specified chromosomal position and corresponding SNP marker. The paternal genotype is shown in red, and the derived genotype for the proband is depicted for deletions along the length of chromosome 18.

Table 2. Specific intervals, size of copy number variants, and genes in the 18q region for case 2

Genomic region [hg19]	Size, Mb	RefSeq genes in the region	Classification
18q21.32(56820380_57837890)×1	1.0	*SEC11C, GRP, RAX, CPLX4, LMAN1, CCBE1, PMAIP1*	VUS
18q21.32q21.33(58585693_59284852)×4	0.7	*CDH20*	VUS
18q21.33(59529458_59593278)×1	0.06	*RNF152*	VUS
18q21.33(60448158_60780827)×1	0.33	*PHLPP1*	VUS
18q21.33q22.1(60957693_66076119)×1	5.1	*BCL2, KDSR, VPS4B, SERPINB5, SERPINB12, SERPINB13, SERPINB4, SERPINB3, SERPINB11, SERPINB7, SERPINB2, SERPINB10, HMSD, SERPINB8, LINC00305, LOC284294, LINC01538, CDH7, CDH19, MIR5011, DSEL, LOC643542*	Likely pathogenic
18q22.2(67520222_67763031)×3	0.24	*CD226, RTTN*	VUS
18q22.2(67807620_68465752)×1	0.65	*RTTN, SOCS6, LOC101927481, LOC101060542, GTSCR1*	VUS
18q22.3(69225340_69297624)×3	0.07	*LINC01541*	
18q22.3(70085574_72826738)×1	2.7	*CBLN2, NETO1, MIR548AV, LOC100505797, LOC400655, LOC100505817, FBXO15, TIMM21, CYB5A, C18orf63, LOC101927606, FAM69C, CNDP2, CNDP1, LINC00909, ZNF407*	VUS
18q23(73,206,898_73,638,515)×1	0.43	*LOC100505853*	Likely benign
18q23(74,196,253_74,237,927)×1	0.04	*ZNF516, LOC101927989, C18orf65*	Likely benign
18q23(74,383,826_74,538,771)×1	0.15	*LOC400661, LOC100131655, ZNF236*	Likely benign
18q23(74,706,420_74,817,142)×1	0.11	*MBP*	VUS
18q23(75,359,543_77,916,234)×1	2.5	*LINC01029, SALL3, ATP9B, NFATC1, LOC284241, CTDP1, KCNG2, PQLC1, HSBP1L1, TXNL4A, RBFA, RBFADN, ADNP2, PARD6G-AS1, PARD6G*	VUS

VUS, variant of uncertain significance.

2010; Kloosterman et al., 2011, 2012; Liu et al., 2011; Genesio et al., 2013, 2015; Gu et al., 2013; Kloosterman and Cuppen, 2013; Fontana et al., 2014; Nazaryan et al., 2014; Plaisancié et al., 2014; de Pagter et al., 2015; Gamba et al., 2015; Macera et al., 2015; Wang et al., 2015; Weckselblatt et al., 2015; Anderson et al., 2016; Bertelsen et al., 2016; Burnside et al., 2016; Del Rey et al., 2016; Masset et al., 2016; Fukami et al., 2017; Sabatini et al., 2018; Gudipati et al., 2019; Koltsova et al., 2019; Nazaryan-Petersen et al., 2019; Zepeda-Mendoza and Morton, 2019; Ader et al., 2020].

In each of our patients, conventional cytogenetics suggested simple deletions. This highlights the limited resolution of G-band analysis in these types of complex rearrangements, and suggests that some previously reported cases of simple deletions could in fact have unrecognized chromoanagenesis events. In these 2 cases, genomic microarray analysis further revealed the presence of numerous breaks with several copy number gains and losses in regions of chromosome 14q and 18q, respectively. While the presence of duplications and triplications is a hallmark of chromoanasynthesis, the specific underlying DNA repair mechanism is currently undetermined in our cases, and thus we describe these rearrangements as being suggestive of the more encompassing descriptor germline chromoanagenesis. Undoubtedly, with the increasingly wide application of high-resolution molecular diagnostic methods such as next-generation sequencing in routine clinical practice, the detection and molecular characterization of such unbalanced catastrophic rearrangements in the germline will likely increase in the future.

Germline complex rearrangements can be stably transmitted from carrier parents or be the result of de novo events arising in either maternal or paternal germlines [Gruchy et al., 2010; Gu et al., 2013; de Pagter et al., 2015; Weckselblatt et al., 2015; Bertelsen et al., 2016; Del Rey et al., 2016; Collins et al., 2017; Grochowski et al., 2018; Sabatini et al., 2018; Gudipati et al., 2019]. While a preferential bias for paternal origin has been described in germline chromothripsis-mediated events, no parental preference has so far been described for chromoanasynthesis-derived rearrangements [Kloosterman et al., 2011, 2012; Liu et al., 2011; Weckselblatt and Rudd, 2015; Weckselblatt et al., 2015; Collins et al., 2017; Fukami et al., 2017; Zepeda-Mendoza and Morton, 2019]. In the current study, the complex rearrangements in both cases were determined to be paternal in origin. Indeed, further data need to be accrued to determine whether a parental bias exists for other forms of chromoanagenesis-compatible germline complex genomic rearrangements.

The patients in this study presented with variable phenotypes including developmental delay, hypotonia, and

seizures. While the pathogenic nature of the rearrangement is evident in each case, a clear genotype-phenotype association was difficult to assess given the complexity of the rearrangements. Undoubtedly, the clinical consequence of any form of complex rearrangement is dependent on dosage effects of the genes located in the regions of copy number change, position effects related to the shuffling of regulatory elements, and the possible disruption of genes located at the breakpoints. Furthermore, the rare cases of phenotypically normal individuals harboring complex rearrangements highlight the additional influence of factors such as variable expressivity, incomplete penetrance, recessive inheritance, and the manifestation of sub-clinical phenotypes. The contributions of each of these effects in complex chromosomal rearrangements will be an important area for future investigation.

In conclusion, this report describes 2 patients with complex chromosome rearrangements having features characteristic of germline chromoanagenesis, most consistent with the subcategory of chromoanasynthesis. The rearranged chromosomes were of paternal origin in both patients, a result which may help elucidate whether preferential parental bias exists broadly in constitutional chromoanagenesis events or is specific to chromothripsis. Future studies such as breakpoint characterization by next-generation sequencing would provide a more detailed assessment of the structural rearrangements in these cases to further characterize the underlying repair mechanisms.

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Statement of Ethics

Written informed consent was obtained from each patient's guardian/parents to participate in this research through our institutional research ethics board protocol (IRB #1011003014).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

P.A. and A.M.B. drafted the manuscript. P.A.M. and P.A. generated and analyzed the data. A.M.B. and J.C.H. contributed to the review and interpretation of the data. P.A., J.C.H., G.H.V., and A.M.B. contributed to the critical review of the manuscript. All authors read and approved the final manuscript.

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