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Copy Number Variants in a Population-Based Investigation of Klippel–Trenaunay Syndrome

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Abstract

Klippel–Trenaunay syndrome (KTS) is a rare congenital vascular disorder that is thought to occur sporadically; however, reports of familial occurrence suggest a genetic component. We examined KTS cases to identify novel, potentially causal copy number variants (CNVs). We identified 17 KTS cases from all live-births occurring in New York (1998–2010). Extracted DNA was genotyped using Illumina microarrays and CNVs were called using PennCNV software. CNVs selected for follow-up had 10 single nucleotide polymorphisms (SNPs) and minimal overlap with in-house controls or controls from the Database of Genomic Variants. We identified 15 candidate CNVs in seven cases; among them a deletion in two cases within transcripts of *HDAC9*, a histone deacetylase essential for angiogenic sprouting of endothelial cells. One of them also had a duplication upstream of *SALL3*, a transcription factor essential for embryonic development that inhibits *DNMT3A*, a DNA methyltransferase responsible for embryonic de novo DNA methylation. Another case had a duplication spanning *ING5*, a histone acetylation regulator active during embryogenesis. We identified rare genetic variants related to chromatin modification which may have a key role in regulating vascular development during embryo-genesis. Further investigation of their implications in the pathogenesis of KTS is warranted.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

angiogenesis; chromatin modification; copy number variant; *DNMT3A*; *HDAC9*; *ING5*; Klippel–Trenaunay syndrome; *SALL3*

INTRODUCTION

Klippel–Trenaunay syndrome (KTS) is a rare congenital vascular disorder affecting approximately one in 100,000 live births [Lorda-Sanchez et al., 1998]. It is characterized by: (i) cutaneous vascular malformations of the capillary (i.e., port-wine stains), venous or lymphatic vessels; (ii) disturbed growth (most commonly hyperplasia) of the affected extremity; and (iii) limb anomalies including polydactyly, syndactyly, and hip dislocation [Oduber et al., 2008]. A more detailed description of the clinical features of KTS in included in Supplementary Material 2. Gastrointestinal or genitourinary hemorrhage in patients with visceral involvement can cause serious morbidity and mortality [Cha et al., 2005]. Rarely, pulmonary embolism and hemorrhage due to intracranial arteriovenous malformations can be fatal [Sadiq et al., 2014]. Odd phenotypes of the syndrome may also occur. Rarely, hypotrophy instead of hypertrophy (inverse KTS) [Danarti et al., 2007], or mixed patterns of increased and decreased growth occur [Ruggieri et al., 2014].

Most KTS cases are assumed to be sporadic; however, several studies have reported familial occurrence [Aelvoet et al., 1992]. Isolated vascular malformations or isolated hemihypertrophy have been identified in family members of KTS patients, suggesting that it might be an inherited syndrome with phenotypic variability or incomplete penetrance [Aelvoet et al., 1992; Craven and Wright, 1995; Lorda-Sanchez et al., 1998]. KTS has also been reported in association with chromosomal anomalies, including balanced translocations and a supernumerary chromosome 18, as well as a susceptibility variant in the angiogenic factor *VG5Q/AGGF1* [Whelan et al., 1995; Wang et al., 2001; Tian et al., 2004; Timur et al., 2004].

The goal of this study was to identify novel, potentially causal, copy number variants (CNVs) that would enhance our understanding of the genetic mechanisms producing KTS.

MATERIALS AND METHODS

Cases

New York State (NYS) has a mandatory birth defects reporting program. Hospitals and physicians are required to report to the state's Congenital Malformation Registry (CMR) children born or living in NYS who have been diagnosed with a congenital malformation before 2 years of age. Each birth defect is coded according to the expanded British Paediatric Association (BPA) coding system. KTS is coded under congenital malformation syndromes involving limbs (BPA code 759.840); therefore, cases with both this specific code in the NYS CMR database and a narrative description of KTS were included. We also searched the database for "Klippel–Trenaunay and "Klippel–Trenaunay Weber" to ensure that no cases were missed. After reviewing the search results and excluding chromosomal abnormalities or other known syndromes, we identified 17 KTS cases from all live births

occurring in NYS from January 1, 1998 through December 31, 2010. The clinical information that was provided by the CMR is available in supplementary material 1 (Supplementary Table SIII).

Medical and demographic data extracted from birth certificates for cases and mothers were compared with those for the population of all live births occurring in NYS (n = 3,264,041) during the same time interval. Statistical analyses were performed with t-test or Fisher's exacttest, where applicable. All cases were de-identified by assignment of a random identification number and all personally identifying data were removed before data analysis and genotyping. This study was approved by the NYS Department of Health (IRB 07–007) and the Office of Human Subjects Research Protection (OHSRP#3687).

Genotyping

Seventeen KTS cases were genotyped. DNA was extracted from two 3 mm dried blood spot (DBS) punches using a laboratory developed method [Saavedra-Matiz et al., 2013], yielding approximately 200–1,200 ng of DNA per case for array genotyping. Samples were genotyped at the Genetic Resources Core Facility, Johns Hopkins University School of Medicine, using Illumina Human-Omni2.5–8_v1 bead arrays and the Infinium HD assay protocol. Quality control samples from unaffected individuals, including four from DBS (one in duplicate) and two HapMap samples, in duplicate, were run concurrently. Illumina GenomeStudio v2011.1 was used for data analysis with a genotype no-call threshold set at <0.15. Genotypes were clustered based on data which included a total of 108 DBS from three birth defect case groups and four DBS from unaffected subjects. Genotypes and clusters were manually reviewed, re-clustered, edited, and excluded where appropriate according to parameters and quality control metrics as described in Illumina's Infinium Genotyping Data Analysis Technical Note [Illumina, 2014]. A total of 2,283,873 autosomal markers were included in the CNV analysis. The average KTS sample call rate SD (range) was 99.90% 0.001 (99.42–99.90%) and the mean log R ratio deviation was 0.125 0.027 (0.093–0.177). Single nucleotide polymorphism (SNP) genotype reproducibility over the three pairs of duplicate samples was 99.99%. One KTS case initially failed genotyping but was successfully rerun from newly extracted DNA on the same platform in a different batch (run with samples at Biomedical Genomics Center Facility at the University of Minnesota, using HumanOmni2.5-8v1.1).

CNV Calling and Annotation

In brief, CNVs were imputed using Illumina's PennCNV version 2011/05/03 and cnvPartition algorithm version 3.2.0 and calls were compiled, and annotated. The percent overlap with common variants reported in HapMap [International HapMap 3 Consortium, 2010] and the Children's Hospital of Philadelphia (CHOP) CNV database [Shaikhetal., 2009], andtheDatabase of Genomic Variants (DGV) was calculated and the following were noted: concordance rates between PennCNV and cnvPartition calls, number of cases or controls with an overlapping CNV, and identification of the intersecting genes and transcripts, using GENCODE Genestrack(version 19, December 2013, HAVANA and Ensembl Datasets).

CNV Selection and Prioritization

Candidate CNVs were excluded if they: were also present in our in-house controls (same CNV type and same predicted breakpoints), contained fewer than 10 consecutive SNP probes, were smaller than 20 Kbps in size, had more than 20% overlap with common variants in HapMap and CHOP databases, or had more than 20% overlap with any variant previously detected in other birth defects (including heterotaxy [Rigler et al., 2015]) screened in our laboratory. CNVs that remained after these exclusions were uploaded to DGV using build37/hg19 coordinates and were examined for overlap with known CNVs (DGV release date—2014/10/16). A CNV was selected for follow up if it had less than 50% overlap with variants catalogued in DGV or if it encompassed a gene in the non-overlapped region. Due the low reproducibility of the results of CNV studies using bacterial artificial chromosome (BAC) microarrays [Ylstra et al., 2006], we did not exclude any CNVs reported to overlap with a CNV identified by a BAC microarray. After these exclusions the remaining 15 CNVs were considered as potentially KTS-related and were tested using quantitative real time polymer-ase chain reaction (qPCR).

CNV Validation

Validation studies were performed using two to four TaqMan qPCRassays (Applied Biosystems, Carlsbad, CA) per region on either an ABI 7900HT or an ABI QuantStudio (Supplementary Materials). Validated CNVs were subsequently screened against 188 race/ethnicity-matched unaffected NYS live births using at least one assay per area of interest.

RESULTS

In the NYS population, the birth prevalence of KTS was 1 per 192,000 live births. KTS cases were compared on maternal demographic factors with all live births from NYS without major birth defects (Table I). KTS was observed to be significantly more common in children of underweight mothers (BMI < 18.5) when compared to all other mothers (BMI > 18.5) [RR: 6.62, 95%CI: 1.37–31.85] but not when compared to only normal weight mothers (BMI 18.5–24.9) [RR: 4.19, 95%CI: 0.85–20.78]. We did not identify any differences in maternal age at delivery or race/ethnicity between cases and controls. Additionally, the sex ratio did not differ significantly between cases and controls, and no differences in birth weight or gestational age were identified.

The microarray analysis of all 17 KTS samples resulted in 2,256 PennCNV and 906 cnvPartition calls. After applying the exclusion criteria described in the methods section, we identified 15 candidate CNVs in seven cases. Of those, 12 CNVs in six cases were validated in the laboratory using qPCR. Validated CNVs included four duplications and two heterozygous deletions intersecting genes, two duplications and one heterozygous deletion encompassing at least one GENCODE transcript, and three intergenic heterozygous deletions (Table II). The CNVs ranged in size from 21 Kbps to 600 Kbps.

Three cases had rare CNVs overlapping genes with functions related to chromatin modification. One case (Case ID 1, Table II) had a 72 Kbps heterozygous deletion at 7p21.1 overlapping two different transcripts of *HDAC9* (Fig. 1a and b). A second case (Case ID 2,

Table II) was found to have a partially overlapping deletion during qPCR validations. The first case also had a 600 Kbps duplication at 18q23 upstream of *SALL3*. We also identified a 98 Kbps duplication in a third case (Case ID 3, Table II) overlapping *ING5*. However, a duplication overlapping both TaqMan copy-number assays used for validation (KT_1343_CXVI3RU [custom designed] and Hs01490888_cn) and *ING5* was also found in one control.

DISCUSSION

In this first genome wide investigation of KTS, we identified CNVs in three cases encompassing genes that are involved in chromatin modification. This process is critical in regulating angiogenesis during embryonic development and is known to be associated with several genetic defects [Hendrich and Bickmore, 2001; Ohtani and Dimmeler, 2011]. Interestingly, chromatin modifying genes have been implicated in the pathogenesis of defects with focal and asymmetric growth such as Silver Russell and Beckwith Weide-mann syndromes by altering tissue specific expression of other genes [Bliek et al., 2006; Delaval et al., 2006]. KTS is postulated to be the result of defective vascular development during embryogenesis [Oduber et al., 2008]; therefore, alterations in chromatin modifying genes may contribute to the pathogenesis of the syndrome.

It is noteworthy that in two cases we identified overlapping heterozygous deletions at 7p21.1 that encompass *HDAC9* transcripts. This gene is important in blood vessel growth and proper vascular organization. *HDAC9* is a member of the Class II histone deacetylase (HDAC) family that regulates angiogenesis by modulating the expression of the anti-angiogenic microRNA 17–92 cluster. In endothelial cells, silencing this gene inhibits VEGF-A expression, and reduces vascular growth. *Hdac9* knockout zebrafish and mice embryos display reduced angiogenic sprouting, and defective vascular formation highlighting this gene's importance in embryonic angiogenesis [Kaluza et al., 2013]. *HDAC9* controls the angiogenic activity of endothelial cells at transcription by encoding multiple splice variants which display distinct functional and localization patterns [Petrie et al., 2003]. The deletion found in Case 1 overlaps exon 1 of isoform 6, a variant that seems to have the essential deacetylase domains but is excluded from the nucleus. It also overlaps intron 2 of isoform 8, a variant that is missing the catalytic subunit and probably lacks deacetylase activity (Fig. 1a and b). It has been suggested that nuclear localization and deacetylation activity transcriptionally control angiogenic activity of endothelial cells in vitro. It is still unclear, however, whether *HDAC9* regulates expression of the miRNA 17–92 cluster through histone modification or whether it targets other transcription factors, activators, or repressors of the cluster [Sato, 2013]. During qPCR validations, Case 2 was found to have a heterozygous deletion using validation assay Hs03646420_cn and was normal for assay Hs03638416_cn; since TaqMan copy-number assays target a single genomic location, we can determine that Case 2 carries a deletion that overlaps at chr7:18,267,017 but not its size.

Case 1 also had a 600 Kbps duplication spanning predicted regulatory elements 450 Kbps upstream of *SALL3*, a spalt-like transcription factor expressed in the embryonic mesoderm. Mutations in several members of the spalt-like family are associated with congenital malformations, indicating that it plays an important role in embryogenesis [Parrish et al.,

2004; Barrio and de Celis, 2008]. *SALL3* interacts with *DNMT3A*, a DNA methyltransferase that catalyzes de novo methylation and participates in establishing DNA methylation patterns in early embryogenesis [Okano et al., 1999; Miranda and Jones, 2007]. Experiments in embryonic cells have shown that *SALL3* binds to DNMT3A and inhibits de novo CpG island methylation [Shikauchi et al., 2009]. Interestingly, DNMT3A also interacts with *HDAC1*, another member of the histone deacetylase family, postulated to be involved in angiogenesis [Fuks et al., 2001; Kim et al., 2001].

We also identified a 98 Kbps duplication at 2q37.3 spanning inhibitor of growth 5 (*ING5*). We are the second group to report on a CNV in this region associated with KTS [Puiu et al., 2013]. *ING5* encodes a transcriptional coactivator and a histone acetylation regulator essential for chromatin remodeling and genomic transcription during embryonic development. More particularly, *ING5* is a component of the of MOZ/MORF complex and is necessary for the histone acetylation activity of the complex as it binds to histone H3K4 [Carlson and Glass, 2014; Yang, 2015]. We also found a similar duplication in one healthy control (based on predicted breakpoints, at least 83% overlap with the duplication identified in Case 3). This finding could also indicate that this variant is not pathogenic, or it that it has incomplete penetrance.

Mosaic somatic mutations have been found in KTS [Lorda-Sanchez et al., 1998; Timur et al., 2004; Happle, 2009]. Mutations in *PIK3CA*, a phosphatidylinositol 3-kinase have been implicated in the pathogenesis of KTS and other overgrowth syndromes such as CLOVES and Proteus syndromes [Kurek Kyle et al., 2012; Youssefian et al., 2015; Vahidnezhad et al., 2016]. A noteworthy study reported an association between KTS and somatic mutations in *PIK3CA* [Luks et al., 2015]. Mutations in this gene were identified in affected tissue from cases with KTS and other lymphatic and vascular disorders. Because there was no germline tissue available to these investigators, we investigated whether, this was a germline mutation by searching for copy number variants overlapping *PIK3CA* but did not identify any. Additionally, another somatic mutation in *RASA1*, a negative regulator of the RAS signaling pathway, have also been associated with KTS and other capillary malformations [Whelan et al., 1995; Eerola et al., 2003; Hershkovitz et al., 2008]; this relationship; however, was not confirmed by other studies [Revencu et al., 2013].

One novel finding of our study was the higher prevalence of KTS in offspring of underweight mothers (BMI < 18.5). KTS was found to be significantly more common in children of underweight mothers when compared to all other mothers. Because there is no evidence that being overweight or obese is protective against malformations, we believe that this finding did not reach statistical significance when only normal weight mothers were used as the comparison group due to the smaller sample size. Pre-pregnancy BMI is thought to be an important determinant of fetal programming and development. Maternal obesity is strongly associated with several birth defects. Maternal pre-pregnancy underweight has been associated with gastroschisis [Lam et al., 1999], as well as atrial septal defects and hypospadias although the data for the latter two defects are mixed [Happle, 2009; Rankin et al., 2010; Block et al., 2013; Cai et al., 2014]. The association between maternal underweight and KTS requires confirmation.

Our study has several strengths. To our knowledge, this is the first whole genome investigation of KTS. The large NYS population from which data were drawn allowed us to accumulate enough cases to examine the role of CNVs in the pathogenesis of this syndrome. Additionally, to our knowledge, this is the first population-based investigation of KTS. By comparing cases to three million births without major birth defects we were able to show that underweight mothers had an increased risk of having a child with KTS. One limitation of our study is the lack of parental DNA in order to evaluate whether, the identified CNVs are inherited or de novo. It should be emphasized; however, that these are rare CNVs that are not present in our control populations. Another limitation of our study should also be noted. Although physicians and hospitals are required to report birth defects to the NYS CMR, there is a possibility of under-ascertainment or incorrect coding. KTS may not be diagnosed in the first 2 years of the infant's life and, therefore, not reported to the CMR. Our prevalence was lower than previous studies have reported. We reported birth prevalence. Other studies might have reported prevalence in older children resulting in more complete ascertainment. It should be noted; however, that ascertainment of selected birth defects by the CMR has been shown to be excellent [Honein and Paulozzi, 1999].

In conclusion, we identified several CNVs in genes involved in chromatin modification, a key regulatory process of embryonic vascular development, which suggests that such genes may be involved in the pathogenesis of KTS. We also identified low maternal BMI as a potential risk factor for this syndrome. Although pathogenic CNVs do not appear to be a frequent cause of KTS, our results suggest avenues for future investigation of the role of chromatin modifying genes in KTS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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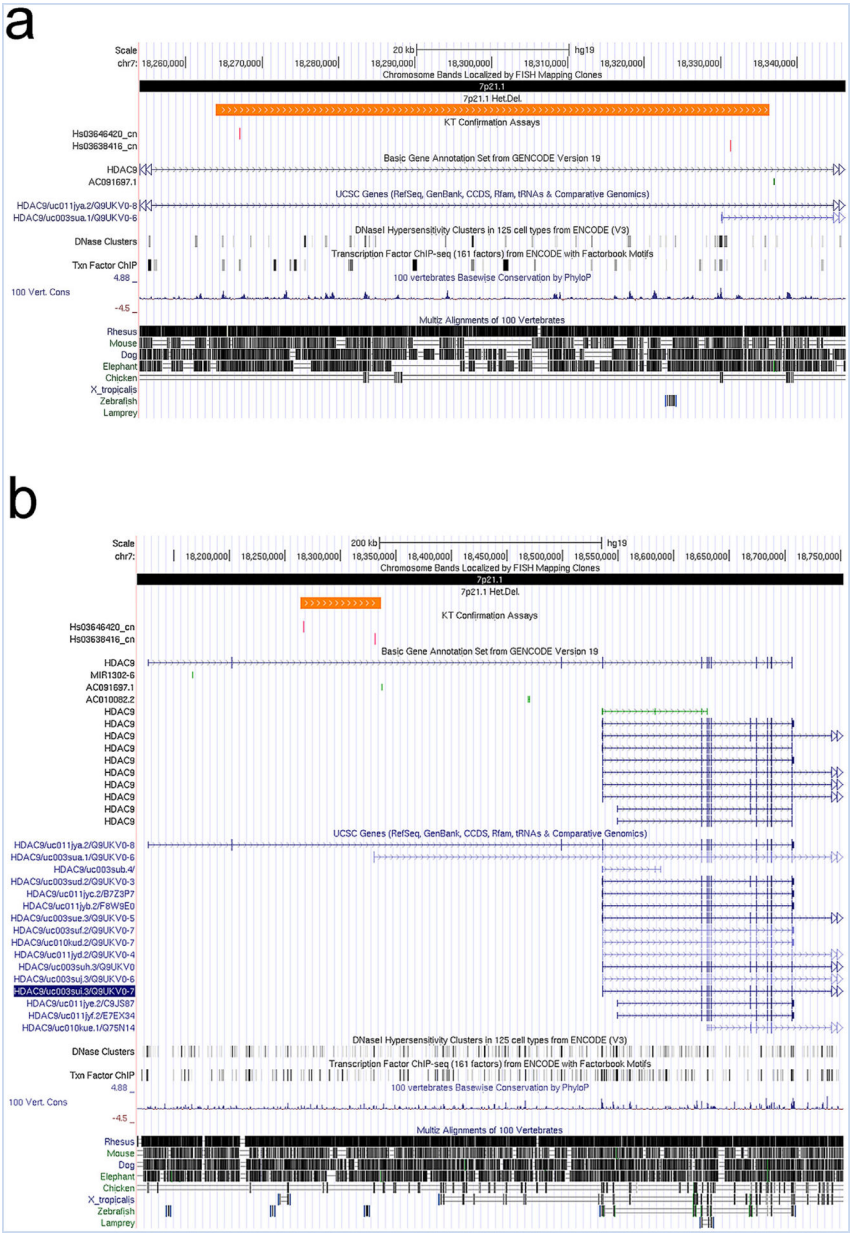

FIG. 1.

HDAC9 isoforms overlapped by the identified 7p21.1 deletion (a). The heterozygous deletion predicted by pennCNV in Case 1 is shown as the orange bar. TaqMan copy-number assays used in qPCR validation are shown as single red lines below the Case 1 CNV. Case 2 was found to be normal at Hs03646420_cn and have a heterozygous deletion at Hs03638416_cn (b). Both figures were exported from the UCSC Genome Browser, hg19 coordinates shown. [Color figure can be viewed at wileyonlinelibrary.com].

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TABLE I.

Selected Characteristics of Klippel–Trenaunay Cases and New York State Live Birth Controls

Characteristic	KTS cases (n = 17)	Live births (n = 3,264,041)	*P*-value
Maternal age, years (%)			0.31
<20	2 (11.8)	241,708 (7.4)	
20–34	9 (52.9)	2,391,031 (73.3)	
≥35	5 (29.4)	630,836 (19.3)	
Missing	1 (5.9)	466 (<0.1)	
Maternal race/ethnicity, n (%)			0.48
Non-Hispanic white	10 (58.8)	1,654,361 (507)	0.61[a]1.00[c]
African American	2 (11.8)	563,134 (17.2)	
Hispanic	3 (17.6)	737,271 (22.6)	0.26[b]0.63[c]
Asian	0 (0)	240,677 (7.4)	
Other	1 (5.9)	62,301 (1.9)	
Missing	1 (5.9)	6,297 (0.2)	
Maternal education, years (%)			0.23
<2	1 (5.9)	633,413 (19.4)	
12	7 (41.2)	894,304 (27.4)	
>2	8 (47.0)	1,701,762 (52.1)	
Missing	1 (5.9)	34,562 (1.1)	
Parity, n (%)			0.81[c]
Nulliparous	6 (35.3)	1,392,043 (42.6)	
Multiparous	10 (58.8)	1,871,998 (57.4)	
Missing	1 (5.9)	0 (0)	
Prepregnancy maternal BMI (kg/m^2)			0.02
<18.5	2 (11.8)	79,815 (2.4)	
18.5–24.9	6 (35.3)	1,004,066 (30.8)	
25–29.9	1 (5.9)	469,577 (14.4)	
≥30	0 (0)	374,566 (11.5)	
Missing	8 (47.0)	1,336,017 (40.9)	
Infant sex, n (%)			0.62[c]

Characteristic	KTS cases (n = 17)	Live births (n = 3,264,041)	*P*-value
Male	7 (41.2)	1,672,803 (51.3)	
Female	9 (52.9)	1,591,224 (487)	
Missing	1 (5.9)	0 (0)	
Infant gestational age (mean days ± SD)	2717 ± 12.2	274.2 ± 18.0	0.57
Infant birth weight (mean grams ± SD)	3272.4 ± 389.9	3285.1 ± 605.2	0.89
Maternal smoking			1.00[c]
No	15 (88.2)	2,969,319 (90.9)	
Yes	1 (5.9)	294722 (9.1)	
Missing	1 (5.9)	0 (0)	

KTS, Klippel–Trenaunay Syndrome.

[a] *P*-value assigned independently to African American versus all other races.

[b] *P*-value assigned independently to Asian versus all other races.

[c] Fisher's Exact Test (Two-sided Pr <= P) *P*-value; all *P*-value calculations exclude missing category.

TABLE II.

Rare CNVs Identified in Klippel–Trenaunay Cases

Locus	Genomic Coordinates[a]	Size (bp)[a]	Type	Case ID	Genes/Transcripts
1p13.2	113,595,229–113,617,151	21,923	Dupl	1	*LRIG2; RP11-31F15.2*
7p14.3	32,040,979–32,082,768	41,790	Het Del	1	*PDE1C*
7p21.1	18,263,964–8,336,338	72,375	Het Del	1[b]	***HDAC9* (uc011jya.2, uc003sua.1)**
18q23	75,522,402–76,163,540[c]	641,139[c]	Dupl	1	*RNA5SP461; RP11-100K18.1; RP11-6F1C19.1; RP11-6F1C19.2, U6*
1q42.3	235,167,295–235,191,818	24,524	Het Del	2[d]	*Intergenic*
6q16.1	93,747,735–93,855,183	107,449	Dupl	2[d]	*RP1-23E21.2*
2q373	242,646,350–242,744,703	98,354	Dupl	3	***ING5***; *D2HGDH; GAL3ST2; NEU4*
5q11.2	56,345,093–56,382,661	37,569	Het Del	4	*Intergenic*
8p12	34,902,928–34,946,452	43,525	Het Del	5	*Intergenic*
20q11.23	35,015,926–35,043,790	27,865	Dupl	5	*DLGAP4*
4q23	101,044,442–101,065,531	21,090	Het Del	6	*AC12115F.1; RP11-15B17.1*
6q12	63,682,426–63,710,643	28,218	Dupl	6	*AL590558.1*

Bolded genes are discussed in the manuscript.

[a] Size and coordinates estimated from array data (pennCNV calls, hg19) unless otherwise noted.

[b] In addition to the 72 Kbps deletion identified in Case 1, Case 2 was found to have a heterozygous deletion (copy number 1) at Chr7:18,267,017 using qPCR.

[c] Size and coordinates from CNV Partition call (hg19) listed because pennCNV call was split into multiple CNVs.

[d] Case 2 initially failed but was rerun with another batch of samples.