Published in final edited form as:
J Med Genet. 2009 September ; 46(9): 626–634. doi:10.1136/jmg.2008.062471.

Genomic duplication resulting in increased copy number of genes encoding the sister chromatid cohesion complex conveys clinical consequences distinct from Cornelia de Lange

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Abstract

Cornelia de Lange Syndrome (CdLS) is a multisystem congenital anomaly disorder. Heterozygous point mutations in three genes (*NIPBL*, *SMC3* and *SMC1A*), encoding components of the sister chromatid cohesion apparatus, are responsible for ~ 50-60% of CdLS cases. Recent studies have revealed a high degree of genomic rearrangements (e.g. deletions and duplications) in the human genome, which result in gene copy number variations (CNV). CNVs have been associated with a wide range of both Mendelian and complex traits including disease phenotypes such as Charcot-

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Marie-Tooth type 1A, Pelizaeus-Merzbacher, Parkinson, Alzheimer, autism and schizophrenia. Increased versus decreased copy number of the same gene can potentially cause either similar or different clinical features. We identified duplications on chromosomes 5 or X using genome wide array Comparative Genomic Hybridization (aCGH). The duplicated regions contain either the *NIPBL* or the *SMC1A* genes. Junction sequences analyses revealed the involvement of three genomic rearrangement mechanisms. The patients share some common features including mental retardation, developmental delay, sleep abnormalities, and crainofacial and limb defects. The systems affected are the same as in CdLS, but clinical manifestations are distinct from CdLS; particularly the absence of the CdLS facial gestalt. Our results confirm the notion that duplication CNV of genes can be a common mechanism for human genetic diseases. Defining the clinical consequences for a specific gene dosage alteration represents a new “reverse genomics” trend in medical genetics that is reciprocal to the traditional approach of delineation of the common clinical phenotype preceding the discovery of the genetic etiology.

Keywords

Copy number changes; CNV; CdLS; *NIPBL*; cohesion complex

Introduction

The human genome has been found to contain a large number of CNVs [1-3], which encompass ∼ 12% of the genome and 14.5% of the genes implicated in human diseases. Copy number alteration of dosage sensitive genes has long been linked to genetic diseases. Charcot-Marie-Tooth disease type 1A [CMT1A, MIM118220] and hereditary neuropathy with liability to pressure palsies [HNPP, MIM162500] result from reciprocal duplication and deletion of the dosage sensitive *PMP22* gene, respectively [4-7]. Deletions and duplications of *PLP1* (proteolipid protein gene) both result in Pelizaeus-Merzbacher disease [PMD, MIM312080], an X-linked dysmyelinating disorder [8-10]. Recent studies have linked CNVs to a broader range of diseases including complex traits, such as Parkinson [11], Alzheimer [12], autism [13-14] and schizophrenia [15-18]. These findings indicate that CNV assessment should be incorporated into the investigations of the genetic bases of diseases including inherited, complex, and sporadic traits [19].

Copy number variation (CNV) results from genomic rearrangements such as duplication and deletion. Three mechanisms have been implicated for the genomic rearrangements including nonallelic homologous recombination (NAHR), nonhomologous end joining (NHEJ) and the recently proposed DNA replication mechanism of Fork Stalling and Template Switching (FoSTeS) [20-24]. NAHR accounts for the majority of the genomic disorders [20] associated with recurrent rearrangements. It occurs mostly between low copy repeats (LCRs) and occasionally using repetitive sequences such as *Alu* or L1 as recombination substrates. NHEJ has been associated with several non-recurrent rearrangements [22]. Further study of the molecular mechanism for non-recurrent rearrangements, especially the complex rearrangements, has recently led to FoSTeS, a replication-based mechanism [24].

Cornelia de Lange Syndrome [CdLS, MIM122470] is a multiple congenital anomaly condition with growth and mental retardation, characteristic facial features, limb defects, neurobehavioral problems, and abnormalities in gastrointestinal, genitourinary and cardiovascular systems [25-27]. The prevalence is estimated to be 1/10,000 [28]. Marked phenotypic variability has been documented. According to the severity of the phenotype, patients have been classified as classical and mild types [25,26,29,30]. Classical facial features consist of synophrys, long eyelashes, long philtrum, thin upper lip and down turned mouth and provide a readily recognizable facial gestalt characteristic of the syndrome.

Heterozygous mutations in the *NIPBL* gene [MIM608667] have been found in ∼ 50% of the CdLS cases [31-36]. Genotype-phenotype correlation analyses revealed that patients carrying *NIPBL* point mutations tend to have a more severe phenotype than mutation negative patients [31,34-36]. Furthermore, there is a trend that for some clinical features, such as growth delay and limb defects, individuals with missense mutations have a milder phenotype than those with truncating mutations. This suggests that *NIPBL* is a dosage sensitive gene. Recently, mutations in two additional cohesin subunit encoding genes, *SMC3* [MIM606062] and *SMC1A* [MIM300040], have been found to contribute to ∼ 5% of CdLS cases [37-38]. These patients demonstrated a mild to moderate phenotype.

Our medical genetics diagnostic laboratory [39-40] identified six cases carrying duplications on chromosome 5 or on the X chromosome with the duplicated regions encompassing *NIPBL* or *SMC1A*. This presented a unique opportunity to study the dosage effect of the genes encoding the subunits of the cohesion complex using a “reverse genomics” approach [41]. These patients have mental retardation, developmental delay, neurobehavioral abnormalities, and craniofacial and limb dysmorphisms. The affected systems are the same as in CdLS. Genome wide 244K oligonucleotide aCGH analyses were performed to refine the size extent, and gene contents of the duplicated intervals and “genomotype” – phenotype correlations were analyzed. Junction sequences were obtained for four patients to study the mechanisms resulting in the duplications. Our results not only confirm that CNV of genes should be considered in searching for etiology of human genetic diseases, but also represent a new “reverse genomics” trend, which is defining the clinical consequences for CNV of a specific genomic interval and attempts to determine the specific gene(s) whose dosage alteration is potentially causative for the phenotype.

Materials and Methods

Subjects and clinical evaluation

Patients were recruited by their referring physicians after our medical genetics diagnostic laboratory initial genomic analyses were performed by the clinical implementation of array Comparative Genomic Hybridization (aCGH) using a targeted array. Clinical phenotype was assessed by experienced medical geneticists. Studies were approved by the Institutional Review Board (IRB) of Baylor College of Medicine and all participants or parental guardians volunteered after informed consent.

Genome wide 244k oligonucleotide aCGH analyses

To perform genome wide 244K oligonucleotide aCGH analyses, genomic DNA was isolated from peripheral blood with a Puregene kit (Gentra Systems). aCGH analyses were performed according to the manufacturer's protocol (Agilent Technologies). DNA samples (~ 2 μg) from patients and gender-matched normal individuals as reference were digested with *Alu*I and *Rsa*I and labeled with Cy5 or Cy3 using a BioPrime array CGH genomic labeling module (Invitrogen). After hybridization, slides were scanned and analyzed for relative gain or loss of fluorescent signals from hybridization of the patient and reference DNAs. Genomic region analyses were performed according to the human reference sequence build 36.1 (UCSC genome browser: http://genome.ucsc.edu) with the software provided by Agilent.

The junctional sequences analyses

Long range PCR primers were designed from the aCGH genomic coordinates to traverse the position of change from gain to “no gain”. Under the assumption of tandem duplication, outwardly facing primers were used to amplify breakpoint junctions. Obtained PCR products were sent for Sanger dideoxy DNA sequencing (SeqWright, Texas).

Results

Chromosomal Microarray Analyses (CMA)

Six unrelated patients were found to harbor gains of a gene encoding a cohesion subunit on either chromosome 5 (*NIPBL*) or the X chromosome (*SMC1A*) by CMA [39] (table 1). The gains were confirmed by interphase fluorescence *in situ* hybridization (FISH) analyses that revealed the extra copy mapped adjacent to the region consistent with interstitial duplication (data not shown). A genomic interval interrogated by BAC clones RP11-317I23, RP11-7M4 (contains *NIPBL*) and RP11-209M10 on chromosome 5 was found to be duplicated in patients 1 and 3. Patients 2 and 5 contain the duplications interrogated by RP11-7M4 and RP11-209M10. A region identified by clones RP11-317I23 and RP11-7M4 is duplicated in patient 4. Patient 6 has a gain in copy number in the proximal region of the short arm of chromosome X covered by BAC clones RP11-416B14, RP11-52N6 (contains *SMC1A*), RP11-70P16. The duplicated regions contain genes in which point mutations result in CdLS. The gains were not detected in the available parental samples (both parents for two patients and 3 maternal samples for three patients). These specific CNVs were not present in the CNV databases of genomic variants (http://projects.tcag.ca/variation/).

High resolution genomic analyses of duplications

In order to analyze the duplications at a higher resolution, we performed genome wide 244k oligonucleotide aCGH analyses (table 1, fig. 1). Patients 1 and 2 appear to have a similar sized 390 kb duplication on chromosome 5, which includes the *NIPBL* gene and exon 3 to the 3′UT region of the *FLJ13231* gene, a gene of unknown function. Patient 3 carries a larger (1.07 Mb) duplication on chromosome 5, which contains *SLC1A3*, *NUP155*, and exons 1 to 9 of the *WDR70* gene in addition to the *NIPBL* and *FLJ13231* genes. *NIPBL* is the only gene located within the 250 kb duplication in patient 4. A 330 kb duplication has

been found in patient 5, which includes exons 39 to 47 of *NIPBL*, *FLJ13231*, *NUP155*, and exons 1 and 2 of *WDR70*. Patient 6 has a 7.14 Mb duplication on X chromosome, which contains about 174 genes (fig. 2). Among them are the *WAS*, *CLCN5*, and *SMC1A* genes responsible for Wiskott-Aldrich syndrome, X-linked nephrolithiasis, and CdLS respectively [37,42,43]. This large duplication also includes the *HUWE1* gene; duplication and point mutation of which have recently been associated with X-linked mental retardation (XLMR) in males [44]. The high resolution 244k Agilent array results are consistent with the CMA findings and further refine the size, extent, and genomic content of duplication and breakpoint region. Additional CNVs identified are provided as a supplementary table.

Breakpoint junctional sequence analyses

To further fine map the duplications and potentially infer rearrangement mechanism(s) from the junctional sequences, we performed breakpoint mapping. The PCR amplification of breakpoint junctions were achieved in 4 out of 6 patients (Pt 2, 3, 4, and 5, fig. 3). Although the oligonucleotide aCGH analyses mapped the breakpoints of patients 1 and 2 to the same genomic interval, the primers that successfully amplified the breakpoint junction of patient 2 failed in patient 1. Therefore these two duplications were not identical. At the breakpoint junction of patient 2, only one base pair was shared between the proximal and distal reference sequences, suggesting the involvement of the non-homologous end joining (NHEJ) mechanism in this rearrangement. The rearrangement in patient 3 was complex. An additional small (176 bp) inverted duplication was observed between the two copies of the large genomic duplication. The distal breakpoint was hypothesized to be caused by non-allelic homologous recombination (NAHR) between two L1 repeats. However, since a 12-bp microhomology (ACAATTAAAAGA) was found, the distal breakpoint was also consistent with our recently proposed DNA replication based FoSTeS (replication Fork Stalling and Template Switching) mechanism while the microhomology could alternatively act in template switching and annealing between replication forks, restarting DNA replication, and generating the DNA rearrangement. No microhomology but the addition of a T was observed in the proximal breakpoint, which was consistent with NHEJ. Similar to patient 2, only one base pair was shared by reference sequences in the breakpoint of patient 4, suggesting that NHEJ caused the duplication. In patient 5, the microhomology of TTT was consistent with NHEJ or FoSTeS×1. In aggregate, the above observations suggest different mechanisms (NHEJ, FoSTeS, and/or NAHR) are responsible for the nonrecurrent genomic duplications that can include the *NIPBL* gene.

Clinical phenotype

Clinical features are summarized in table 2. All patients had developmental delay and mental retardation. The majority of the patients had low birth weight and became overweight in adulthood. Hypotonia and seizures were commonly seen and periventricular leukomalacia (2/2) was observed in two head imaging studies (one CT and one MRI) available. Patients shared some craniofacial dysmorphic features including microbrachycephaly (3/6), frontal bossing (3/6), a short philtrum (4/6), low-set ears (3/6), with one patient having a significant skull deformity (patient 4, fig. 4G and 4H). The patients also had long-fingers (3/6) and neurobehavioral abnormalities including sleep

disturbances (3/6) (table 2). Available individual clinical details are provided as supplementary material.

Discussion

Both increased and decreased copy number of a dosage sensitive gene can cause congenital diseases. This is the case both for autosomal genes and genes on the X chromosome. Reciprocal duplication and deletion of the *PMP22* gene result in CMT1A and HNPP respectively [4-6], both are inherited neuropathies but with distinct clinical presentations. Williams -Beuren syndrome (WBS) and its reciprocal duplication syndrome are the results of deletion and duplication of a region on 7q11.23 [45-48]. WBS is featured by cognitive deficiency with relative preservation of linguistic abilities, neurobehavioral problems, recognizable facial dysmorphism, and cardiac and ophthalmologic abnormalities. Patients with the apparent reciprocal duplication of the WBS region have mild dysmorphisms and a severe delay in expressive language [46-48]. In most cases, the increased dosage from duplication causes a milder phenotype than deletion of the same region as in the Smith-Magenis syndrome [SMS, MIM182290] and its reciprocal duplication syndrome, Potocki-Lupski syndrome [PTLS, MIM610883] [49-50], as well as in the duplication and loss of function of the *MECP2* gene [51-53]. Patients with SMS have characteristic craniofacial, skeletal and neurobehavioral features as well as defects in multiple organ systems. PTLS is featured by mental retardation and behavioral problems including autistic features and sleep apnea. Sometimes both duplication and deletion result in similar phenotype as in Pelizaeus-Merzbacher disease (PMD), an X-linked dysmyelinating disorder [8-10].

Heterozygous mutations of the *NIPBL* or the *SMC1A* genes results in CdLS, which is characterized by mental retardation, developmental delay, neurobehavioral abnormalities, and craniofacial and limb defects [25,26,32,33,37]. We identified six patients with genomic duplications containing either *NIPBL* (N=5) or *SMC1A*. These individuals also have mental retardation, developmental delay, and neurobehavioral abnormalities such as hypotonia and sleep disturbances (table 2). Only a limited number of patients have been observed [five that have duplications including *NIPBL* and only one whose large > 7 Mb duplication includes *SMC1A*] and the clinical manifestations may be complicated by *in utero* exposures (maternal diabetes for patient 4 and ethanol during the pregnancy of patient 5) and different sized genomic duplications. Nevertheless, the patients clearly do not have CdLS and thus we can conclude that duplications harboring genes for the cohesion complex present with phenotypes distinct from those observed with haploinsufficiency effects for the same genes.

Common craniofacial features include frontal bossing, broad nasal root, low set ears, short philtrum and high palate. The shared facial defects make the patients appear similar to each other (fig. 4), but with this limited patient set from different ages no recognizable facial gestalt is apparent. The craniofacial features are different from those that are typical for CdLS, the latter of which include synophrys, long eyelashes, long philtrum, thin upper lip and down turned mouth [25-26]. However, some facial abnormalities have been frequently observed in patients with CdLS, such as low set ears, high palate, and ocular anomalies. Patients with duplication of the gene for cohesion complex proteins also have increased weight and long fingers, which are opposite to characteristics observed in CdLS.

NIPBL is the only gene duplicated in patient 4 (table 1). But it is hard to determine whether the severe skull deformity is the result of the duplication. The skull deformity complicated the assessment of the phenotype. Further follow up will hopefully reveal the effect of the duplication. Patients 1 and 2 have a similar sized genomic duplication, which results in the duplication of *NIPBL* and exon 3 and 3′ untranslated region of *FLJ13231*, a gene of unknown function. Both *NIPBL* and *FLJ13231* are duplicated in patient 3, who has clinical features similar to patients 1 and 2. The duplication in patient 5 results in the duplication of *FLJ13231* and *Nup155*, as in patient 3. But the *NIPBL* gene is only partially duplicated, which most likely does not result in the increased expression of *NIPBL*. Indeed, the phenotype in patient 5 is the least similar to other patients, and the phenotypic analysis is likely complicated by the maternal ingestion of alcohol in the first trimester. Based on these observations, *NIPBL* is likely the major dosage sensitive gene for the phenotype, but more patients are necessary to further delineate the effects of the increased dosage of the *NIPBL* gene.

Patient 6 has a large duplication containing the *SMC1A* gene. Her phenotype is similar to patient 1 and 2; however, with only one patient available it is difficult to assess if other genes also contribute to the phenotype. The duplication contains the *WAS* gene, responsible for Wiskott-Aldrich syndrome, featured by recurrent infections and eczema [42], that have also been observed in patient 6. Whether this is the result of the duplication of *WAS* needs further investigation. This female patient is also duplicated for the *HUWE1* gene, duplication or point mutation of which has been recently associated with XLMR in males. Five of the families segregating XLMR in association with duplication of *HUWE1* also include the *SMC1A* within their duplications [44]. However, these males are described as having nonsyndromic MR [44]. For all six patients reported herein with duplications including genes encoding subunits of the cohesin complex, other genomic CNV were identified (supplementary table), but most are present in the database of genomic variants. Whether such variants can sometimes modify the ultimate phenotype is unclear at this time.

Three mechanisms have been implicated for the genomic rearrangements including NAHR, NHEJ and recently proposed FoSTeS [20-24]. NAHR occurs mostly between low copy repeats (LCRs) and occasionally through repetitive sequences such as *Alu* or L1. Sequence analysis surrounding the duplication breakpoints failed to detect any LCRs (data not shown) and our junction analyses revealed the involvement of all three mechanisms. Despite the similar duplication in aCGH analyses for patients 1 and 2, our results indicate that a more complicated rearrangement may have occurred in patient 1.

A tremendous amount of copy number variations (CNVs) has been identified in the human genome recently [1-3]. CNVs have been associated with multiple congenital diseases as well as some complex traits such as autism and schizophrenia [13-15]. Our findings confirm the notion that CNV of genes involved in dominant Mendelian traits could be a common mechanism for human genetic diseases. We further document that phenotypes conveyed by duplication of dosage sensitive gene may be milder than those caused by loss-of-function due to haploinsufficiency caused by deletion or point mutation. The distinct phenotype conveyed by duplication versus deletion may challenge discovery of duplication alleles at a given disease locus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the patients and their families for their generous participation. This work was supported in part by a grant from the National Institute of Child Health and Human development (PO1 HD39420) and the Baylor College of Medicine Mental Retardation Research Center (MRRC) (HD 2406407).

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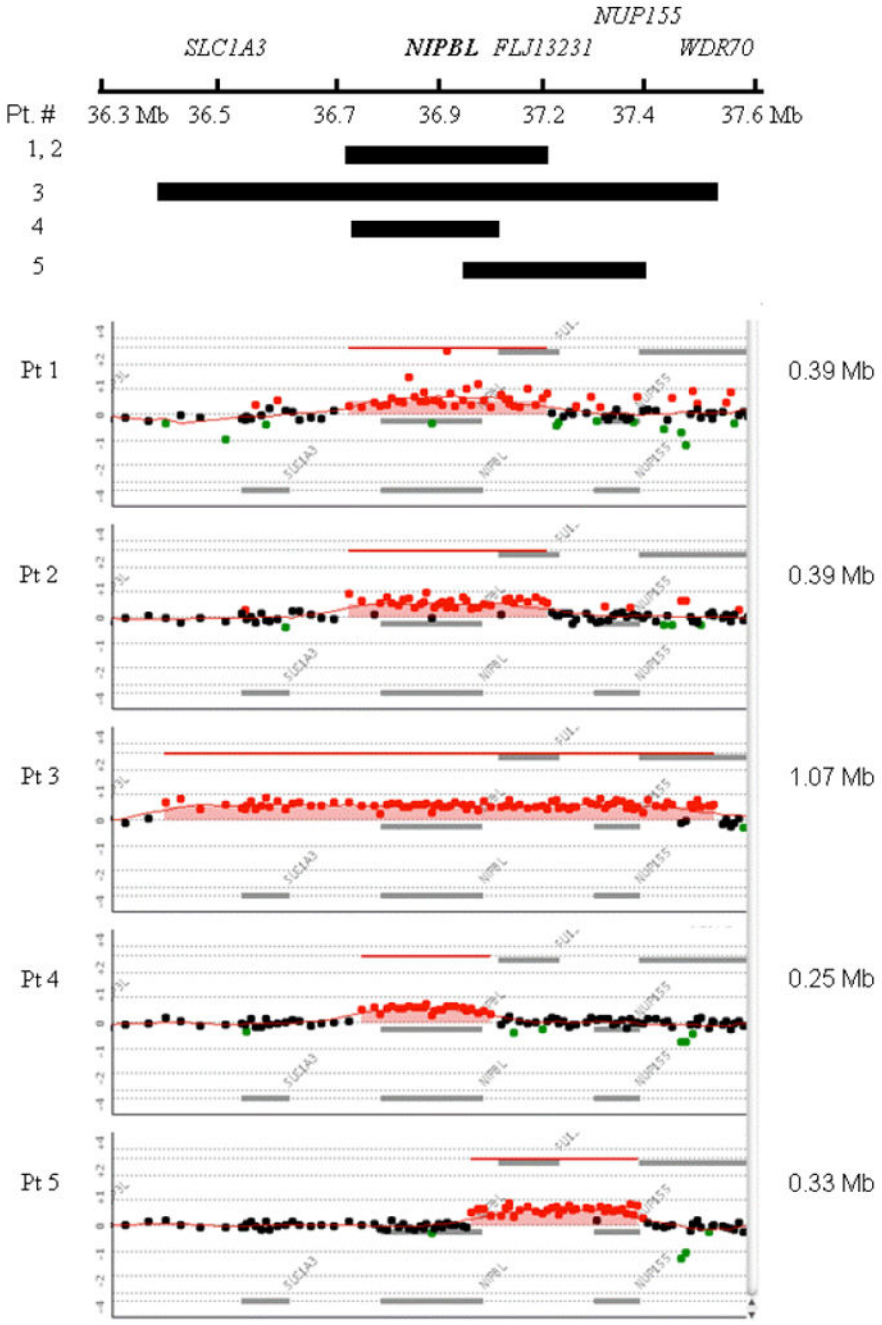

Figure 1.

aCGH analyses for patients. Map of the *NIPBL* region on chromosome 5 (top) with the extent of the gains detected shown by horizontal bars. Below are the aCGH results from the Agilent 244K arrays. A gain, as evidenced by $\log_2$ ratios >0.5, is denoted by red dots with each signifying the relative signal from a single interrogating oligonucleotide. To the right are given the sizes of the individual duplications.

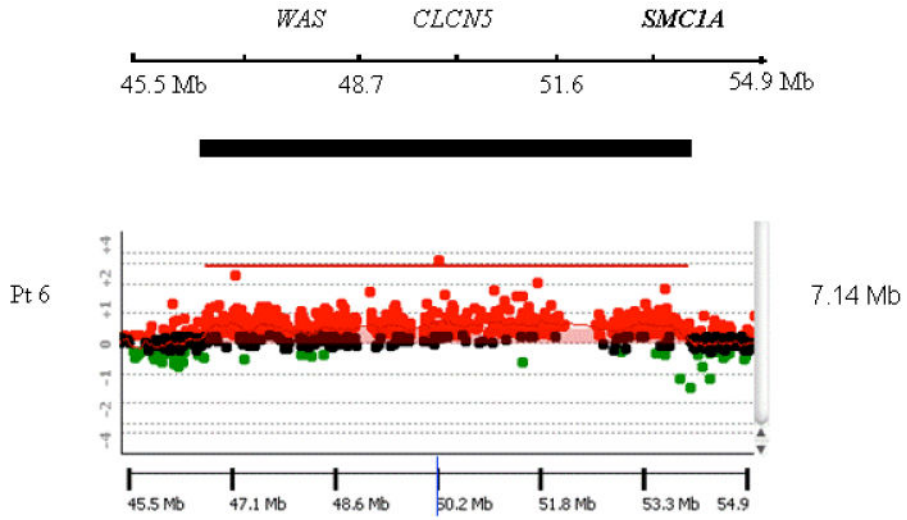

Figure 2.

aCGH analyses for patient 6. Shown on top is the *SMC1A* region on chromosome X. The gain detected is shown by a horizontal bar. Below is the aCGH result with the size given at the right.

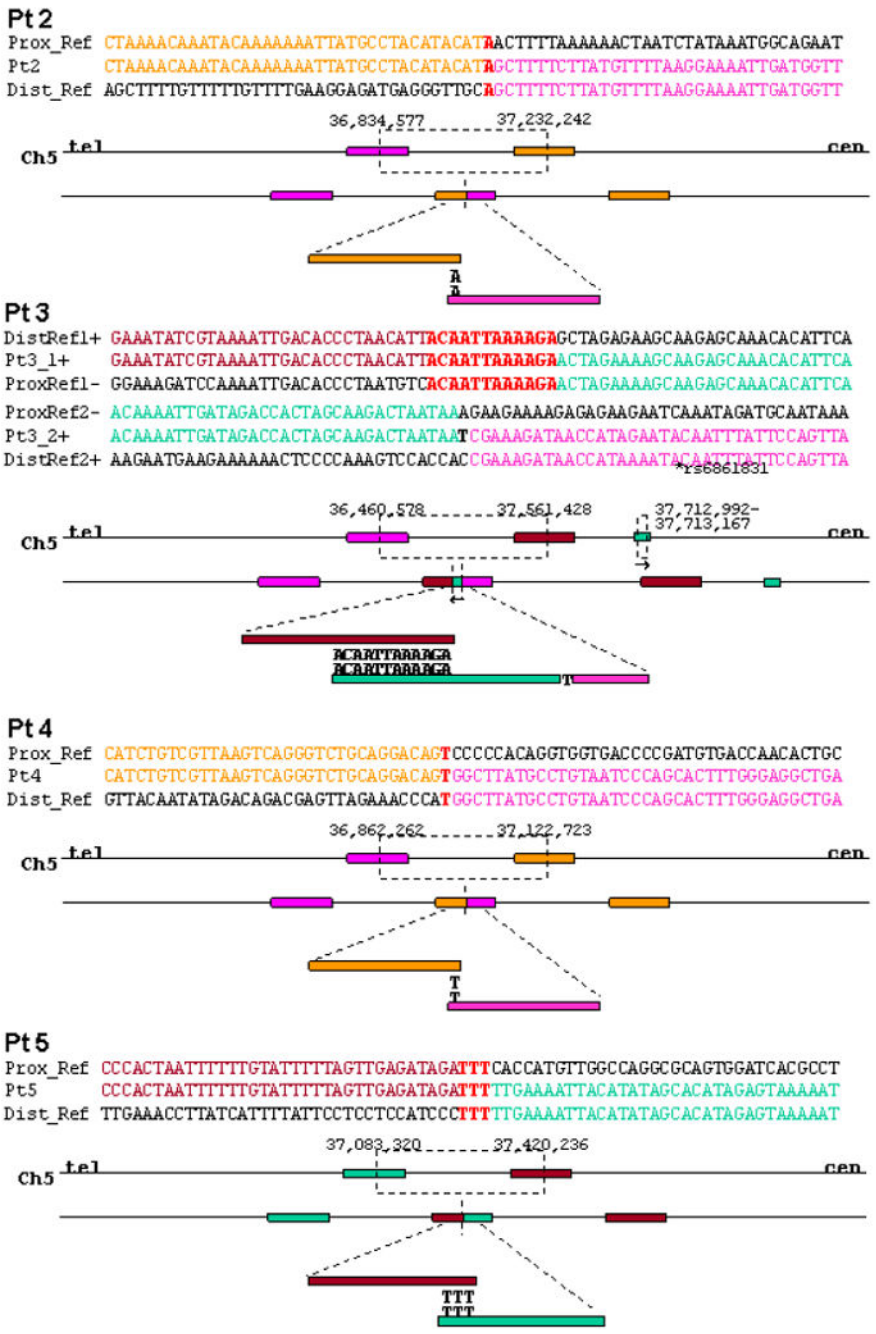

Figure 3.

Breakpoint sequence analyses. The amplification of breakpoint junctions were achieved in four patients (Pt 2, 3, 4, and 5). The duplication regions were shown in dash rectangles. The breakpoint coordinates (NCBI Build 36.1) were also shown. The 1-bp mismatch (marked by asterisk) between the breakpoint junction sequence of patient 3 and its distal reference sequence resulted from the SNP-rs6861831.

Figure 4.

Photographs of patients. Front and side views of patients 1 (A, B), 2 (C, D) and 5 (I, J); (E) patient 3 at 25 years of age; (F) patient 3 at 12 years of age; K: patient 6. Note the mild craniofacial defects. G and H: CT scan of skull of patient 3. Note the severe skull deformity. We have obtained the written consent from the patient or his/her legal guardian for publication of the images in print and online.

Table 1

Genomic regions duplicated in patients

Pt	chromosome	Duplication Size (Mb)	Genomic region*	Gene(s)	BACs detecting duplication in CMA analysis	CMA version
1	5	0.39	36845462-37231819	***NIPBL***, *FLJ13231* (exon 3-3′UT)	RP11-317I23, RP11-7M4, RP11-209M10	Version 5 BAC array
2	5	0.39	36845462-37231819	***NIPBL***, *FLJ13231* (exon 3-3′UT)	RP11-7M4, RP11-209M10	Version 6 oligo array
3	5	1.07	36487985-37556112	*SLC1A3*, ***NIPBL***, *FLJ13231*, *NUP155*, *WDR70* (exon 1-9)	RP11-317I23, RP11-7M4, RP11-209M10	Version 6 oligo array
4	5	0.25	36870689-37120908	***NIPBL***	RP11-317I23, RP11-7M4	Version 6 oligo array
5	5	0.33	37084359-37417228	***NIPBL*** (exon 39-47), *FLJ13231*, *NUP155*, *WDR70* (exon 1-2)	RP11-7M4, RP11-209M10	Version 6 oligo array
6	X	7.14	46824905-53961694	∼174 genes including *WAS*, *CLCN5*, ***SMC1A***	RP11-416B14, RP11-52N6, RP11-70P16	Version 6 BAC array

*based on the 244K aCGH data

Table 2

Clinical features of patients

Patient	1	2	3	4	5	6	CdLS*
Gender	F	F	F	M	F	F	
Age	18 years	6 years	30 years	8 months	5 years	6 years	
Birth height	NA	3%	3%	NA	NA	normal	low
Birth weight	NA	<5%	<5%	2.29 kg	50%	normal	low
Height	normal	normal	162.6 cm	<5%	<3%	normal	Short stature
Weight	> 95%	90%	59.2 Kg	<5%	50%	> 98%	low
Mental retardation	+	+	+	NA	+	+	+
Developmental delay	+	+	+	+	+	+	+ (global psychomotor delay)
Behavioral abnormalities	Sleep disturbance Self-injurious Repetitive stereotypic movements Obsessive-compulsive behavior	Sleep disturbance	Suggested diagnosis of pervasive disorder	NA	No particular abnormalities	Sleep abnormalities Aggressive Pain insensitivity Anxiety	Self-injurious Autistic-like features Hyperactivity Attention disorder Anxiety Compulsive disorders Sleep disturbance
Hypotonia	-	+	NA	+	-	+	+
Seizures	-	staring spells	NA	no	+	+, staring spells	+
Facial features and skull defects	Macrocephaly Broad, tall forehead Bitemporal narrowing	Frontal bossing	Frontal bossing Biparietal narrowing	Multiple suture craniosynostosis turricephaly brachiocephaly	Large flat forehead	Microcephaly	Microbrachycephaly

Ocular anomalies	Short and upslanting palpebral fissures Exotropia	Astigmatism Strabismus	Hypotelorism Right epicanthic fold	Hypertelorism Proptosis	Short palpebral fissures	Long palpebral fissures Excess skin infraorbitally Esotropia	Palpebral ptosis Myopia Nystagmus
Nose	Bulbous nasal tip Broad nasal root	flat, wide base			Groove between nasal root and the face	Tubular nose with a pointed tip Prominent columella	Small nose Depressed nasal bridge Anterverted nostrils
Mouth	Short philtrum Down-turned corners of mouth Prognathism Narrow palate	Short philtrum	Short philtrum Micrognathia Highly arched palate	High-arched palate	Poorly defined philtrum Several incisors missing	High palate	Micrognathia Crescent-shaped mouth Long philtrum Thin lips High arched palate
Ears	Low set	Low set, posteriorly rotated, cupped		Low set, small, and simplified in shape	Small pits anterior to the ear helixes Hearing disorder		Low set Posteriorly augulated Hearing disorder
Hairs, eyebrow			Low posterior hairline	Hair scant Absent eyebrows		Thick hair Heavy, long eyebrows	Low posterior hairline Synophrys Long eyelashes
Limb defects	Large hands, feet Very long fingers Right thumb: ulnar deviation Single palmer crease	Long fingers Right knee genu valgum	Normal	Single transverse palmar crease on the left hand	Short 5th fingers and a prominent hockey stick crease	Long gracile fingers 5th finger clinodactyly Pes planus	Small hands and feet Short digits Phocomelia Oligodactyly 5th finger clinodactyly Proximally placed thumbs Single palmer crease
Other defects	Scoliosis			Agenesis of the corpus callosum		Hirsutism Eczema Recurrent sinus and ear infections	Hirsutism Gastroesophageal reflux Heart defects

						Vesicoureteral reflux (VUR)	Vesicoureteral reflux (VUR)

F:female; M: male; NA: information not available

* common features for CdLS20,21,23,54