RESEARCH REVIEW

Special Cases in Cornelia de Lange Syndrome: The Spanish Experience

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Cornelia de Lange Syndrome (CdLS) is an autosomal dominant (*NIPBL*, *SMC3*, and *RAD21*) or X-linked (*SMC1A* and *HDAC8*) disorder, characterized by distinctive craniofacial appearance, growth retardation, intellectual disability, and limb anomalies. In 2005, the Spanish CdLS Reference Center was started and now we have more than 270 cases in our database. In this special issue, we describe some of the unique or atypical patients studied by our group, whose clinical features have contributed to the expansion of the CdLS classical phenotype, helping clinicians to diagnose it. We include the case of a male with unilateral tibial hypoplasia and peroneal agenesis who had a mutation in *NIPBL*; we also describe one patient with a mutation in *NIPBL* and somatic mosaicism identified by new generation sequencing techniques; we also include one patient with CdLS and Turner syndrome; and last, an interesting patient with a duplication of the *SMC1A* gene. Finally, we make a short review of the splicing mutations we have found in *NIPBL* regarding the new knowledge on the physiological variants of the gene. © 2016 Wiley Periodicals, Inc.

KEY WORDS: Cornelia de Lange syndrome; *NIPBL*; somatic mosaicism; splicing variants

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INTRODUCTION

In 2005, a triple collaboration between the Department of Pediatrics of the Hospital Clínico Universitario "Lozano Blesa" in Zaragoza, the Unit of Clinical Genetics and Functional Genomics of the University of Zaragoza Medical School, and the Spanish Association of Cornelia de Lange Syndrome (CdLS) began and enabled the launch in the Spain's National CdLS Reference Center which today has expanded to another cohesinopathies, and related syndromes. Since then, more than 270 patients have been registered in our database.

Cornelia de Lange syndrome is a developmental malformation syndrome characterized by distinctive facial features, growth and psychomotor retardation, intellectual disability, limb malformations, and hirsutism. In addition, congenital malformations or functional malformations in different systems may be present, such as gastrointestinal, cardiovascular, nervous system, eye, and hearing [Kline et al., 2007]. It is estimated that the prevalence of CdLS is 1/45,000–1/62,000 live births [Barisic et al., 2008], although this figure is likely underestimated due to the existence of individuals with mild or atypical phenotypes who have not been diagnosed.

To date, five causative genes have been identified: *NIPBL*, *SMC1A*, *SMC3*, *RAD21*, and *HDAC8*, all sharing encoding structural or regulatory components of the cohesin complex [Mannini et al., 2013]. This complex is essential for the coordinated segregation of chromosomes, DNA repair, regulation of gene expression, and maintenance of chromatiń s structure [Liu and Krantz, 2008; Mehta et al., 2013]. However, up to 20–30% of patients still remain undiagnosed, probably due to the existence of other causal genes, somatic mosaicism, and copy-number variants (CNVs) [Ramos et al., 2015; Deardorff et al., 2016]. Furthermore, phenotypic variations are so wide that patients with mutations in still unknown causal genes remain undiagnosed. In this paper, we describe some of atypical cases studied by our group [Wierzba et al., 2012; Baquero-Montoya et al., 2014a,b,c; Gil-Rodríguez et al., 2014]. We hope this knowledge will help to expand the clinical boundaries of CdLS and contribute to facilitate its diagnosis. Finally, we include a short review of the splicing mutations we found in *NIPBL* regarding the new knowledge on the physiological variants of the gene [Teresa-Rodrigo et al., 2014, 2016].

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SPECIAL CASES

Patient With Severe Ipsilateral Lower Limb Malformation and *NIPBL* Mutation

This is a patient from Senegal with CdLS phenotype and atypical limb defects. Parents were consanguineous and he was diagnosed right after birth due to his typical CdLS facies. He had unilateral limb reduction which included oligodactyly (second and fourth fingers) in the right hand and tibial hypoplasia with peroneal agenesis in the right leg. The lower limb malformation had not been previously described and confirmed the involvement of the proximal shaft—in the distal limb development—of these patients. Interestingly, the limbs of the left side were completely normal (Table I; Fig. 1, P1 i–iv) [Baquero-Montoya et al., 2014a]. Surprisingly, despite the serious unilateral limb reduction defect the patient had only mild neurocognitive impairment [Gillis et al., 2004; Kline et al., 2007]. The Sanger sequencing identified a c.6,647A>G mutation in *NIPBL*, which resulted in the change of a highly conserved amino acid p. (Tyr2,216Cys) [Baquero-Montoya et al., 2014a].

From the clinical standpoint, body asymmetries are usually indicators of somatic mosaicism [Youssoufian and Pyeritz, 2002], but, in this case, pyrosequencing studies ruled out a mosaic mutation. In addition, the finding of hypoplasia of the tibia and agenesis of fibula prompted the complementary studies, such as full exome, CGH-Array, and MLPA (Multiplex ligation-dependent probe amplification), which excluded the involvement of other potential causative genes [Baquero-Montoya et al., 2014a].

The findings in this case suggest that the hypoplasia of the tibia and the fibula agenesis are likely due to the missense mutation of the *NIPBL* gene, and serve to expand the CdLS phenotype spectrum.

Somatic Mosaicism in a Patient With *NIPBL* Mutation Identified by Next Generation Sequencing

This case is an example of how the new techniques of Next Generation Sequencing (NGS) help in the diagnosis of a patient with CdLS and somatic mosaicism of the *NIPBL* gene. Initially, the patient was studied by classical Sanger sequencing with negative results. However, the discovery of a mutation by the new techniques served to strength the hypothesis that undiagnosed patients may have mutations in a mosaic state, and that the most appropriate approach for diagnosis is to use NGS, or study of DNA from cells of oral mucosa epithelial cells or fibroblasts [Baquero-Montoya et al., 2014b].

TABLE I. Clinical and Molecular Features of Four Patients With CdLS

Patient	P1	P2	P3	P4
Gene mutated	*NIPBL*	*NIPBL*	*NIPBL* and TS	*SMC1A* duplication
Exon	39	39	35	
cDNA mutation*	c.6,647A>G	c.6,647A>C	c.1,445_1,448delGAGA	
Effect on mRNA/protein	p.Tyr2,216Cys	p.Tyr2,216Ser	p.Arg482Asnfs*20	
Type of mutation	Missense	Missense/ somatic mosaicism	Frameshift	Duplication Xp11.22 region ~1.1 Mb, encompassing the *SMC1A* gene
Gender	M	F	F	M
Year of birth	2009	2007	2007	1995
Birth weight (g)	2.230	2.260	1.350	2.330
Length at birth (cm)	44.5	43	43	44
OFC at birth (cm)	31	30.5	25	31
APGAR score	9/10	9/10	7/9	9/10
IUGR	+	+	+	+
Postnatal growth retardation	+	+	+	+
Craniofacial features	Synophrys, arched eyebrows,long eyelashed, depressed nasal bridge with anteverted nostrils Micrognatia Microcephaly	Synophrys, arched eyebrows,long eyelashed, depressed nasal bridge with anteverted nostrils Microcephaly	Synophrys, arched eyebrows, long eyelashes, hypertelorism, depressed nasal bridge, anteverted nares, long and flat philtrum Microretrognathia, **Short and webbed neck**	Synophrys, arched and bushy eyebrows, long eyelashes, broad nasal bridge, prominent philtrum, Microretrognathia
Limb malformations	Oligodactyly in right hand Right leg showed tibial agenesis and fibular hypoplasia	Small hands and feet (<3rd centile) Proximally placed thumbs, bilateral fifth finger clinodactyly	Small hands and feet, fifth finger clinodactyly, proximally placed thumbs single palmar crease and hip dysplasia	Brachydactyly, bilateral fifth finger clinodactyly, small hands and feet (< 3rd centile), and hyperextensible joints
Psychomotor delay	+	+	+	+
Intellectual disability	+	+	+	+
Hirsutism	+	+	+	+
Cardiovascular abnormality	ASD	Foramen ovale Aberrant subclavian artery	ASD, VSD, and PS	–
Gastroesophageal reflux	–	+	+	–
ENT-Hearing	–	–	Sensorineural hypoacusia	–
Genitourinay problems	–	–	–	Vesicoureteral reflux Atrophy on the left kidney Bladder diverticulum
Feeding problems in infancy	+	+	+	–

TABLE I. (*Continued*)

Patient	P1	P2	P3	P4
Seizures	–	–	–	+
Other findings	–	Tuberous angioma on the neck	**Congenital bilateral glaucoma and retinopathy Lymphedema Cubitus valgus**	–
Clinical severity	Moderate (score 16)	Moderate (score 19)	Severe (score 24)	Mild (score 11)

Numbering is based on *NIPBL* cDNA sequences (RefSeq numbers NM_133433), starting from the first nucleotide of the ORF. Nomenclature is according to den Dunnen and Antonarakis and to the Human Genome Variation Society Mutation Nomenclature Recommendations. **In bold**, symptoms associated to Turner Syndrome (TS). (+), present; (–), not present; N/A, not available; OFC, occipito-frontal circumference; IUGR, intrauterine growth retardation; ENT, ear, nose, and throat; CNS, central nervous system; ASD, atrial septal defect; VSD, ventricular septal defect, PS, pulmonary stenosis.

However, the discovery of a mutation by the new techniques served to strength the hypothesis that undiagnosed patients may have mutations in a mosaic state, and that the most appropriate approach for diagnosis is to use NGS, or study of DNA from cells of oral mucosa epithelial cells or fibroblasts.

The patient was the first child of non-consanguineous Spanish parents. Prenatal ultrasound studies during the first trimester of pregnancy detected intrauterine growth retardation. Physical examination in the neonatal period showed anterior and posterior low insertion of hair, synophrys with arched eyebrows, long and thick eyelashes, depressed nasal root and anteverted nostrils, low-set posteriorly rotated ears, long and smooth philtrum, thin upper lip with down-turned lips, high arched palate, micrognathia, hirsutism, and generalized cutis marmorata. She also had severe gastroesophageal reflux and delayed psychomotor development that allow to classify as a classic case of CdLS with severe phenotype (Table I; Fig. 1, P2, v–viii) [Baquero-Montoya et al., 2014b].

Molecular studies by Sanger sequencing of *NIPBL*, *SMC1A*, *SMC3*, *HDAC8*, and *RAD21* were negative. However, molecular analysis of the exome identified a de novo missense mutation in exon 39 of *NIPBL*: c.6647A>G; p. (Tyr2261Ser). The mutant allele was detected in 5 of the 21 (24%) sequence readings. A re-evaluation of the Sanger sequencing results allowed to visualize a weak signal in the chromatograms. The result suggested a mutation in mosaic state. Then, we performed pyrosequencing studies in order to quantify the mutated allele and confirm the biallelic *NIPBL* transcript expression. The results revealed specific heterozygosity values in each tissue analyzed. The mutated allele was present in $10.5\% \pm 0.2\%$ of the sequences in leukocytes DNA, in $23.4\% \pm 0.5\%$ of buccal epithelial cells, and $46.6 \pm 1.1\%$% of fibroblasts. Therefore, we confirmed the biallelic expression of the mutation and we could demonstrate a higher expression in fibroblasts (43.0%), than in peripheral blood leukocytes ($0.6\% \pm 0.9\%$) [Baquero-Montoya et al., 2014b].

This case is similar to previously reported cases [Huisman et al., 2013], and points at that a significant number of patients with CdLS and negative molecular diagnosis may represent somatic mosaicisms of *NIPBL*. In addition, our results suggest that the Sanger sequencing technique is itself unable to detect mutations in mosaic state below a certain proportion of affected alleles [Rohlin et al., 2009]. The hypothesis that leukocytes are not the most suitable cells for these diagnostic studies, and that epithelial cells from oral mucosa are a better option, is supported by our case. It is therefore advisable to use, when it is possible, NGS techniques, such as specific panels or full exome studies due to their higher sensitivity.

Cornelia de Lange Syndrome and Mosaic Turner Syndrome in the Same Individual

This patient had CdLS, confirmed by the identification of a mutation in *NIPBL*, and Turner syndrome (TS) with mosaic karyotype (45,X/46,XX). The coincidence of two genetic conditions and the observation of that, ontogenetically, the mutation in the *NIPBL* gene appeared earlier than the aneuploidy of the X chromosome, support the hypothesis that mutations in the cohesin complex could be related to alterations in chromosome segregation [Wierzba et al., 2012; Gil-Rodríguez et al., 2014]. Previously, only two patients with CdLS and TS had been reported [Klosovskiĭ et al., 1968; Hoppman-Chaney et al., 2011].

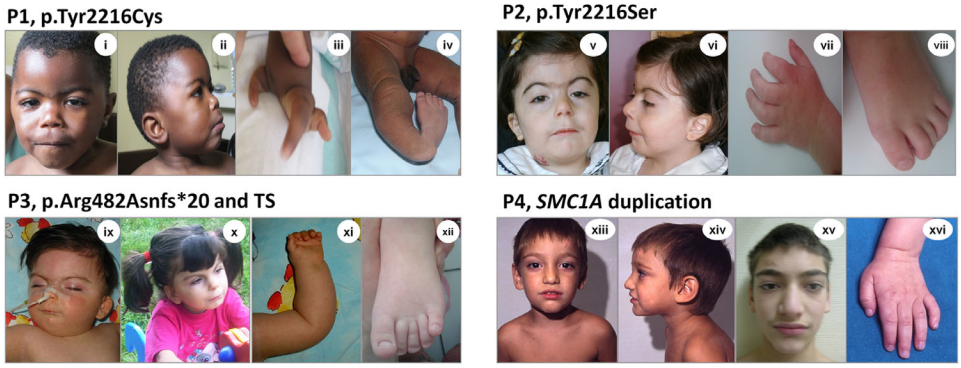

Figure 1. Phenotype of the patients. Patient 1 (P1). (i–ii) Frontal and side-view of the patient at the 3 years of age; right hand (iii) and lower limbs (iv) in the first week of life, respectively. Patient 2 (P2). (v–vi) Frontal and side-view of the proband at the age of 4 years and 11 months; (vii–viii) left hand and left foot at the age of 5 years. Patient 3 (P3). (ix–x) Frontal view of the patient at 9 months of age and 4 years and 8 months, respectively; (xi) right arm at the 9 months and (xii) left foot at 3 years of age. Patient 4 (P4). Frontal view of the proband at the age of (xiii) 4 and (xv) 16. Side-view (xiv) and left hand (xvi) at the 4 years of age.

The female patient was the first child of healthy non consanguineous parents, and no family history of birth defects or intellectual disability. Prenatal ultrasound studies showed intrauterine growth retardation in the second trimester of gestation. Physical examination in the newborn period showed typical features of CdLS: distinctive facial phenotype, low birth weight and hirsutism, and typical features of Turner syndrome: distal lower limb lymphedema, webbed neck, cubitus valgus, nail hypoplasia, short sternum, and increased intermammilar distance. Additional features reported in both CdLS and TS were also noticed over time: short stature, congenital glaucoma, retinopathy, low-set ears, high arched palate, cardiovascular anomalies, congenital hip dysplasia, sensorineural hearing loss, speech delay, poor sucking, and psychomotor retardation (Table I; Fig. 1, P3 were also found, ix–xii) [Wierzba et al., 2012; Gil-Rodríguez et al., 2014].

Molecular analysis identified a frameshift mutation in exon 9 of *NIPBL* (c.1,445_1,448delGAGA), that likely generated a nonfunctional truncated protein (p. (Arg482Asnfs*20). On the other hand, FISH analysis showed monosomy of the X chromosome in 28% of peripheral blood leukocytes and in 7% of cells from oral mucosa. However, the *NIPBL* mutation was identified in both tissues, which virtually ruled out a somatic mosaicism. These findings suggested that, ontogenetically, the mutation in *NIPBL* appeared earlier than the X chromosome aneuploidy, and the question was whether the mutation had affected the normal mechanism of segregation of the chromosomes (canonical function of cohesin), and produced the monosomy. This hypothesis may provide an explanation for the aneuploidy in our case since the most common cause of mosaicism is non disjunction in early postzygotic mitotic divisions. [Gil-Rodríguez et al., 2014]. However, there is no enough experimental evidence to prove that the loss of function of *NIPBL* cause segregation defects in cell models or in cells from patients with CdLS. Therefore, new studies need to be performed to confirm this hypothesis.

Clinical Phenotype of a Patient With *SMC1A* Duplication

This patient is the second reported case of a duplication of the gene *SMC1A*. This patient, despite having a multisystem involvement and molecular findings consistent with the Cornelia de Lange spectrum, has different craniofacial features which require inclusion in a larger group of disorders of the cohesin complex (cohesinopathies) [Baquero-Montoya et al., 2014c].

The patient was initially assessed due to prenatal growth retardation and multiple congenital anomalies. Craniofacial features reported during the neonatal period included synophrys, arched bushy eyebrows, long eyelashes, broad nasal bridge with bulbous nose, prominent philtrum, carp-shaped mouth, micro-retrognathia, the low insertion of the hair, and generalized hirsutism. Renal anomalies included left cortical atrophy, vesicoureteral reflux, and a bladder diverticulum. At the age of 5 years, he had psychomotor retardation and developed generalized tonic-clonic seizures. At age 16, the anthropometric parameters continued to be under the third percentile for age and gender. Neurocognitively, he had an IQ of 57 and behavioral disturbances such as extreme shyness, autism spectrum disorder, and attention deficit hyperactivity disorder. In his clinical history, the diagnosis of CdLS was never mentioned. He had a high-resolution karyotype that showed the existence of a supernumerary unidentified marker (47,XY,+mar), a CGH-Array, that identified a gain in the number of copies the Xp11.22 region of approximately 1.1 Mb, and encompassing 17 genes, including *SMC1A* [Baquero-Montoya et al., 2014c].

Initially, the duplication of 17 genes could explain the broad clinical

spectrum of the patient, however, data from the literature on the involved genes, and the prevalence of neurological and cognitive symptoms, suggested that the clinical phenotype was more likely linked to the involvement of the *SMC1A* gene. Furthermore, this finding supported the hypothesis that brain is the most sensitive organ to disruption by cohesins. On the other hand, the milder clinical manifestations were more likely to be caused by gene duplication. Our results were also similar to the previously reported patient with *SMC1A* duplication, [Yan et al., 2009] and the four patients who carried similar chromosomal markers registered in databases. They all had mild phenotypes related to the cohesinopathies clinical spectrum, but they could not be diagnosed as CdLS patients [Le Caignec et al., 2003; Santos et al., 2007; Baker et al., 2010; Liehr et al., 2013].

Therefore, it is recommended that in patients with intellectual disability, pre- and postnatal growth retardation and musculoskeletal malformations of unclear origin, genes of the cohesin complex should be studied both by analysis of mutations, and copy number. It seems also appropriate to include patients with duplication in *SMC1A* in a broader clinical CdLS spectrum, known as cohesinopathies.

Therefore, it is recommended that in patients with intellectual disability, pre- and postnatal growth retardation and musculoskeletal malformations of unclear origin, genes of the cohesin complex should be studied both by analysis of mutations and copy number. It seems also appropriate to include patients with duplication in SMC1A in a broader clinical CdLS spectrum, known as cohesinopathies.

PHYSIOLOGICAL AND PATHOLOGICAL SPLICING OF THE *NIPBL* GENE

A large number of studies had demonstrated that more than 90% of human genes undergo alternative splicing and that mutations altering splicing are more common than previously thought. Recently, the possibility of correcting the defective splicing was brought as an important therapeutic strategy in the field of rare diseases [Tazi et al., 2009]. Therefore, the study of physiological splicing of the *NIPBL* gene and the detailed characterization of its splicing mutations could be of great value in the study of CdLS.

One step forward in this direction has been the systematic search for physiological splicing variants in *NIPBL*. By using this approach, the presence of the NIPBL B-isoform protein in human leukocytes was demonstrated in fetal tissues [Tonkin et al., 2004]. Furthermore, we have identified four new splicing variants, ΔE10, ΔE12, ΔE33,34, and B', due to the loss of exons 10, 12, 33 + 34, and 45 (Fig. 2A). Although the large size of the gene prevented us from checking for transcripts by the combination of these variants, its knowledge is essential to properly assess the impact of splicing mutations [Teresa-Rodrigo et al., 2014].

Of the more than 350 known point mutations in *NIPBL*, 70 are located in splice-sites (Fig. 2B), which means that almost 20% of the mutations could alter the splicing of the gene. However, these mutations have traditionally been very rarely studied at the RNA level, so the actual impact of most of them is unknown. Notably, while nonsense mutations tend to accumulate in the first half of the gene and the missense in the second half [Strachan, 2005; Pié et al., 2010], splicing mutations appear to have a random distribution pattern. Mutations have been identified in almost all introns of the *NIPBL* gene, although in most of them, only one or two mutations have been reported and never more than four in the same intron (Fig. 2B).

Given the fundamental mechanism by which the splicing mutations work, generating aberrant transcripts that alter or not the reading frame, genotype-phenotype correlations in patients with such mutations could be specifically studied. In the first published systematic characterization of splicing mutations in *NIPBL*, we analyzed eight splicing mutations in 12 patients with CdLS, seven of them previously unreported [Teresa-Rodrigo et al., 2014]. We were able to show that the most severe phenotypes are associated with mutations that generate aberrant frameshift transcripts. However, the description of variants in *NIPBL* (i.e., c.6.109-3T>C) [Gillis et al., 2004; Selicorni et al., 2007] as a possible mutation splicing, that was later ruled out in RNA studies [Teresa-Rodrigo et al., 2014], supports the need for experimental confirmation, even if it is costly and time-consuming. It is also possible that some patients with CdLS who are still undiagnosed, bear undetected splicing mutations because they are located far away from the canonical positions. To date, the two deepest intronic mutations described in the *NIPBL* gene have been c.5,329-15A>G, that, according to functional studies affects the branch point and produce an aberrant transcript with the loss of exon 28 and maintenance of reading frame, and the c.6,344del (−13)_ (−8), located in the polypyrimidine stretch, which generates a transcript with loss of exon 37 and rupture of the reading frame [Teresa-Rodrigo et al., 2016]. Again, the mutation that generates an aberrant frameshift transcript is associated with a more severe phenotype, and produces a marked decrease in the expression of the gene. This decrease indicates a haploinsufficiency, which correlates well with the severe phenotype of the patient and highlights the importance of considering the intronic variants as

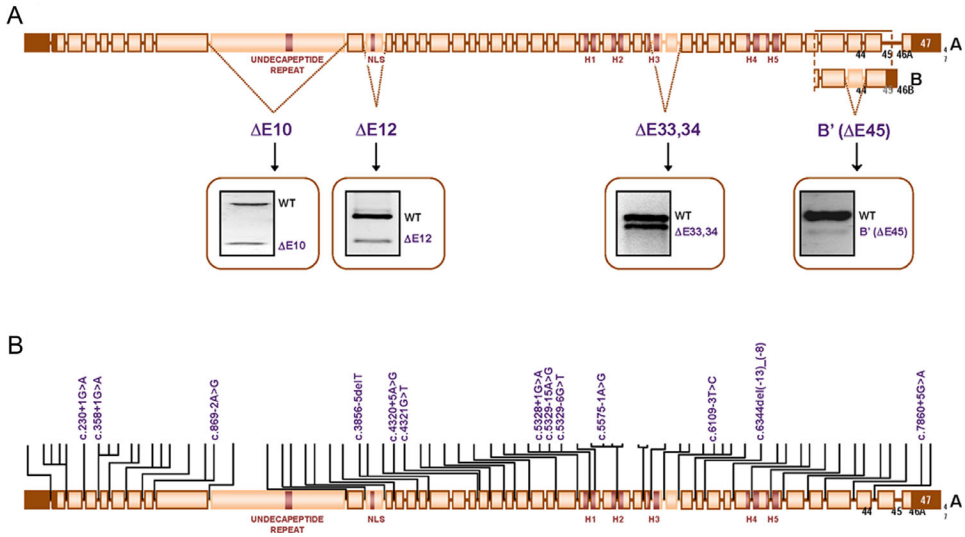

Figure 2. **(A)** Schematic representation of the *NIPBL* gene and its physiological splicing variants. Four new variants have been identified: Δ10, Δ12, Δ33,34, and B', due to the loss of exons 10, 12, 33 + 34, and 45 on isoform B, respectively. (**B**) Distribution of all the splicing mutations reported along *NIPBL*. The mutations studied by our group are highlighted. Note the uniform distribution of splicing mutations.

possible causative mutations by performing molecular diagnosis in these patients.

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