ARTICLE

Intragenic and large *NIPBL* rearrangements revealed by MLPA in Cornelia de Lange patients

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Cornelia de Lange syndrome (CdLS) is a rare multisystemic congenital anomaly disorder that is characterised by intellectual disability and growth retardation, congenital heart defects, intestinal anomalies, facial dysmorphism (including synophyris and high arched eyebrows) and limb reduction defects. Mutations in three cohesin-associated genes encoding a key regulator (*NIPBL*, chr 5p13.2) and one structural component of the cohesin ring (*SMC1A*, chr Xp11) occur in about 65% of CdLS patients. *NIPBL* is the major causative gene, and accounts for 40–60% of CdLS patients as shown by a number of mutational screening studies that indicate a wide mutational repertoire of mainly small deletions and point mutations. Only a few data are available concerning the occurrence of large *NIPBL* rearrangements or intragenic deletions or duplications involving whole exons. We used multiplex ligation-dependent probe amplification (MLPA) to study 132 CdLS patients negative to the standard mutation *NIPBL* test out of a cohort of 200 CdLS patients. A total of 7 out of 132 patients were found to carry *NIPBL* alterations, including two large gene deletions extending beyond the gene, four intragenic multi- or single-exon deletions and one single-exon duplication. These findings show that MLPA leads to a 5.3% increase in the detection of mutations when used in addition to the standard *NIPBL* scan, and contributes *per se* to the molecular diagnosis of 3.5% (7/200) of clinically diagnosed CdLS patients. It is recommended that MLPA be included in the CdLS diagnostic flow chart.

INTRODUCTION

Cornelia de Lange syndrome (CdLS; MIM#122470, 300590) is a multi-system malformation/intellectual disability disorder caused by the developmental and post-natal defects of the cohesin pathway.[1–3] Alterations in the nipped-B-like *NIPBL* (MIM#608667) and structural maintenance of chromosomes 1A *SMC1A* (#300040) genes, which encode a cohesin regulator and a structural subunit of the cohesin complex, respectively, account for 40–60 and 5% of CdLS patients,[4–7] but other causative genes are thought to be involved in CdLS patients who are negative for mutations in the known genes. Consequently, especially in the case of the major *NIPBL* gene, the diagnostic test should be refined by means of a multi-method approach and sequencing extended to the non-coding regions in order to reveal the entire fraction of patients with gene mutation and identify definitive negative patients suitable for next-generation sequencing aimed at detecting novel genetic determinants. Genomic and gene-targeted techniques such as array-comparative genomic hybridisation (a-CGH) and multiplex ligation-dependent probe amplification (MLPA) can reveal some *NIPBL* imbalances overlooked by the standard test, although both techniques have only been sporadically used to screen CdLS patients. In patients with a CdLS-like clinical diagnosis who are negative to *NIPBL* scan, a-CGH has mainly revealed *de novo* imbalances in various genomic regions that maybe too large to identify new CdLS genes.[8–10] Given the length of the *NIPBL* gene and the plausible failure of its splicing mechanism, MLPA undoubtedly seems to be the most promising additional means of optimising the diagnostic mutation rate. However, only three MLPA-detected CdLS patients have so far been described in the literature, one of which was fortuitously found in the context of a study aimed at evaluating the increased DNA damage sensitivity of CdLS syndrome cell lines.[11] The other two patients, one with an intragenic deletion encompassing *NIPBL* exons 41–42 and the other with a large deletion affecting the last 13 exons of *NIPBL* and extending beyond it, were identified from cohorts of 50 and 11 CdLS patients, respectively, who were negative to the standard gene test.[12,13] In order to evaluate the fraction of CdLS patients undiagnosed by *NIPBL* denaturing high-performance liquid chromatography (DHPLC)/sequencing, we used MLPA to process systematically 132 out of 200 CdLS patients negative to the test. Seven of the negative patients were found to bear a previously undetected *NIPBL* alteration. We also characterised at cDNA level three intragenic *NIPBL* rearrangements and refined the genomic mapping of two large deletions, respectively, extending to the 5′UTR and 3′UTR of the *NIPBL* gene.

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METHODS

Subjects

A cohort of 200 patients with a clinical diagnosis of CdLS (108 males and 92 females, ranging from newborn to adult age) has so far been referred to our Institute for the genetic screening of the *NIPBL* and *SMC1A* genes. The CdLS patients of our cohort had been evaluated by different clinical geneticists, all experienced in CdLS, who used the parameters of Kline *et al*[14] and, whenever possible, were given an overall clinical score. Peripheral blood samples were obtained from all the patients following their or their parents' informed consent.

DHPLC and direct sequencing

The genomic DNAs underwent the mutational screening of the *NIPBL* gene (RefSeq NM_133433) by means of DHPLC using intronic primers flanking the whole coding sequence and the predicted promoter region (http://www.cbs.dtu.dk/services/Promoter/, promoter 2.0 prediction) set up for polymerase chain reaction (PCR) and DHPLC temperature conditions. Heteroduplex fragments underwent direct sequencing. The detailed protocol is available on request.

MLPA analysis

The SALSA P141/P142 *NIPBL* MLPA kit (MRC-Holland, Amsterdam, The Netherlands) was used in accordance with the manufacturer's instructions. The parents' DNA was analysed to establish the *de novo* origin of the rearrangements. cDNA was analysed when sufficient material was available. Genomic real-time qPCR was carried out to validate the MLPA-detected duplication.

SNP analysis

The segregation of the centromeric-to-telomeric SNPs rs12658985, rs11740860, rs10035888, rs10491374, rs1004202, rs10042632 and rs10036454 from the parents to the probands was checked by sequencing in order to define the largest *NIPBL* deletion.

cDNA analysis of mutant alleles

Total RNA was extracted from fresh peripheral blood or lymphoblastoid cell lines using the Trizol procedure (Life Technologies, Grand Island, NY, USA). cDNA for RT-PCR was obtained using the SuperScript First-Strand Synthesis System (Invitrogen Ltd, Inchinnan Business Park, UK), with the primers being selected by means of Primer Express software. Primers amplifying a product from untranslated exon 1 to exon 3 (fw: 5′-ATTATAGTCTCTCGCCAC-3′ and rev: 5′-GTAGTTGTAGCAGGTAAAG-3′), from exon 24 to exon 28 (fw: 5′-ACTGTTGCTGCACGGCTAA-3′ and rev: 5′-CTCGTTGCATATCAAGCCTTG-3′) and from exon 2 to exon 4 (fw: 5′-ACTGTTGCTGCACGGCTAA-3′ and rev: 5′-CTCGTTGCATATCAAGCCTTG-3′) were used to characterise deletions. The PCR products from the cDNAs were loaded on 3% agarose gel, eluted by means of GFX (Gel Heathcare, Buckinghamshire, UK) and sequenced using the above primers in order to evaluate the presence and ratio of the aberrant transcripts in comparison with the wild type.

Real-time qPCR assay

For the real-time qPCR assay of genomic DNA, the SYBR Green protocol (SYBR GREEN, PCR Master Mix, Applied Biosystem, Warrington, UK) was applied using primers located within exon 32 (fw: 5′-CTTTGATTCTTTTCATCACCCTTA-3′ and rev: 5′-GCAGTTGCTCAAACCAGTCA-3′). Amplicons were run as triplicate in separate tubes to allow the quantification of the *NIPBL* gene, normalised to the endogenous *PGK1* (phosphoglycerate kinase 1) gene and amplified using primers fw: 5′-GAAGTGGAGAAAGCCTGTGCC-3′ and rev: 5′-TTCTTCCTCCACATGAAAGCG-3′. The gene copy number of the investigated sample was then estimated using a calibrator sample of normal, healthy control DNA. The results were quantified using the comparative threshold cycle method.

Clone preparation

All of the BAC clones were purchased from Invitrogen S.r. l. DNA was obtained from a single colony grown in 10 ml of LB medium, supplemented with 12.5 μg/ml chloramphenicol (High Performance CCD Camera (COHU,

Poway, CA, USA) and visualised using McProbe software (Applied Imaging, PowerGene, League City, TX, USA)) in accordance with standard procedures.

Cell culture and chromosome preparations

Phytohemagglutinin-stimulated peripheral blood lymphocytes from the samples were set up in culture using Chromosome Kit 'Synchro' (Celbio, Milan, Italy) and modified RPMI (Irvine Scientific, Santa Ana, CA, USA) plus 5% foetal calf serum (Gibco, Grand Island, NY, USA). The cultures were stopped with colchicine after 72 h. The chromosome preparations were obtained using standard methods.

FISH

The BAC probes were labelled with digoxigenin-dUTP (Roche Diagnostic, Mannheim, Germany) using a nick translation kit (Roche Diagnostic). The FISH experiments were performed using standard procedures.[15] The chromosomes were counterstained with DAPI in antifade (Vectashield Mounting medium, Vector Laboratories Inc., Burlingame, CA, USA), and then visualised using a Leitz DM-RB microscope equipped for DAPI and FITC/TRITC epifluorescence optics. The images were captured by means of a CCD camera (Hamamatsu 3CCD Camera, C5810, Hamamatsu City, Japan), and visualised using McProbe software.

a-CGH analysis

Genomic DNA was extracted from probands' and parents' whole blood using the GenElute Blood Genomic DNA kit (Sigma-Aldrich, St Louis, MO, USA) in accordance with the manufacturer's instructions. The reference was pooled DNA from the peripheral blood of 10 healthy donors (Promega, Madison, WI, USA) who were gender-matched to the samples. The genome scan was performed using the Human Genome CGH Microarray Kit 244K (Agilent Technologies, Palo Alto, CA, USA), which consists of ∼236 000 60-mer oligonucleotide probes covering the entire genome at an average spatial resolution of ∼30 kb. The samples were labelled and hybridised following the protocols provided by Agilent. The arrays were analysed using the Agilent Scanner Control (v 7.0) and Feature Extraction software (v 9.5.1). Graphical overviews were obtained using CGH Analytic software (v 4.0.81). A log ratio plot between the test and reference genomic DNA was assigned so that aberrations in test DNA copy numbers at a particular locus were observed as a deviation of the ratio plots from a modal value of 0. Aberration calls were identified using the ADM-2 algorithm. An *in silico* analysis of the observed unbalanced region was made using the March 2006 release of the UCSC Genome Browser (http://genome.ucsc.edu/) and the Database of Genomic Variants (http://projects.tcag.ca/variation).

RESULTS

A cohort of 200 patients ranging from earliest infancy to adult age with a clinical diagnosis of CdLS underwent molecular screening of the *NIPBL* gene. They were divided into three subgroups on the basis of their clinical score (severe > 22, moderate 15–22, and mild < 15) as previously described in the case of 62 of them.[7] *NIPBL* DHPLC and sequencing of the coding and flanking regions plus a portion of the 5′UTR allowed us to identify 68 patients with gene mutations, including those previously described:[7] 29 truncating (19 frameshift and 10 nonsense), 13 missense and 23 splicing mutations, and 2 in-frame deletions affecting the coding and consensus sequence, and 1 mutation in the 5′UTR, for an *NIPBL* mutation rate of 34%. This low rate may be explained by the clinical heterogeneity of this cohort that includes a considerable number of patients with a very mild phenotype. All of the samples that were negative to the standard *NIPBL* scan were processed using the MLPA test in order to evaluate the occurrence of whole exon deletions/duplications. This revealed six deletions: one involving the first 10 exons of *NIPBL* and 2 Mb upstream (patient 1), one whole gene deletion extending 0.5 Mb downstream (patient 7), the intragenic deletion of three exons (25–27) in patient 4, and three
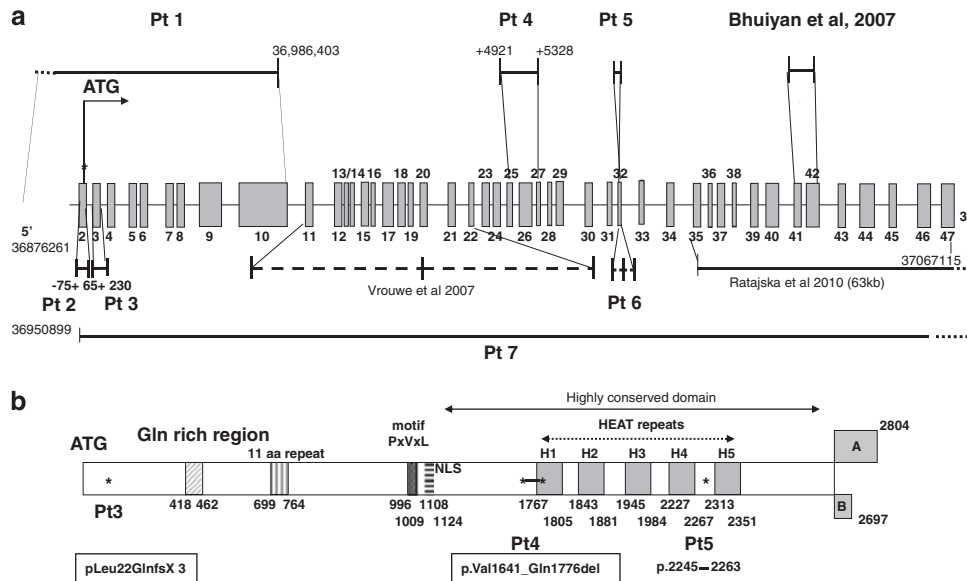
Figure 1 Overall picture of MLPA-detected large deletions/duplications involving the *NIPBL* gene. (**a**) Schematic illustration of the *NIPBL* gene: the black bars mark the genomic deletions in patients 1 and 7 and the skipped exons in patients 2, 3, 4, and 5; the findings relating to two previously described patients in the literature are also included. The dashed bars indicate the duplications of patient 6 and the patient reported by Vrouwe; the large genomic duplications reported by Yan *et al*[16] are not included because they are out of scale. The genomic boundaries of the rearrangements of pts 1 and 7 and cDNA boundaries of pts 2, 3, and 4 are indicated at the ends of each bar. (**b**) Schematic illustration of the NIPBL protein with its putative domains and motifs: the asterisks indicate the localisation of the predicted deleted amino-acid residues (patients 4 and 5) and the protein truncation start site (patient 3).

Table 1 Clinical features of CdLS patients in this study

							Major	Intellectual				Mutation	
		Age,	Facial			Limb	malformations[a]	disability[b]					
Pt	Sex	year	dysmorphism	IUGR	PNGR	reduction			Speech	Medical complications	Clinical score[c]	Localisation	Type
1	M	36	+	+	+	+	−	Severe	Absent	GER, deafness	38 Severe	5′UTR ex 1–10	Large deletion (2 Mb)
2	F	16	+	−	−	−	−	Moderate	Retarded	GER, obesity	20 Mild	Ex 2	Deletion
3	F	3	+	−	+	−	−	Moderate	Absent	GER	18 Mild	Ex 3	Deletion
4	M	4	+	+	+	+	−	Severe	Absent	GER	23 Severe	Ex 25–27	Deletion
5	M	12	+	+	+	−	2 (G; U)	Severe	Absent	Myopia deafness	34 Severe	Ex 32	Deletion
6	M	—	+	−	NA	NA	NA	NA	NA	NA	NA	Ex 32	Duplication
7	F	6	+	+	+	+	1 (B)	Moderate	Retarded	GER, and myopia, full body hirsutism	26 Severe	Ex 2–47 3′UTR	Large deletion (515 kb)
8[d]	F	1, 8	+	−	+	+	2 (C;U)	Severe	NA	GER, full body hirsutism, hypertonic, seizures, apnea	28 Severe	Ex 41–42	Deletion
9[e]	F	14	+	−	+	+	3 (C;K;U)	Severe	Absent	GER	30 Severe	Ex 35–47 3′UTR	Large deletion (63 kb)

Abbreviations: B, brain; G, genitalia; GER, gastroesophageal reflux; GI, gastrointestinal; IUGR, intrauterin growth retardation; NA, not available; PNGR, postnatal growth retardation; U, urinary.
[a]Numbers refer to different apparatus/anatomic sites.
[b]Mild<15; moderate 15–22; severe>22.
[c]According to Kline *et al*.[14]
[d]Bhuiyan *et al*.[12]
[e]Ratajska *et al*.[13]

single exon deletions in patients 2 (exon 2), 3 (exon 3) and 5 (exon 32), and a duplication of exon 32 in patient 6. The parents of all of the patients with deletions were non-carriers; the deletions were considered *de novo*. The identified *NIPBL* rearrangements are shown in Figure 1a, which also includes two previous MLPA-detected intragenic deletions[12,13] and one duplication.[11] Five large genomic duplications on chromosome 5 involving the *NIPBL* gene have also been reported in patients that share some of the features of CdLS but not its facial gestalt.[16] As MLPA does not allow the precise mapping of the rearrangements or predict the coding of a deleted truncating or in-frame protein, we investigated the mutations at transcript level in

three patients for whom biological samples were available (see next section). Figure 1b shows the putative position of the deletions on the protein and the various delangin domains affected. In the case of *NIPBL* whole gene deletion (patient 7), deletion of the first 10 exons (patient 1) or the deletion of exon 2 (patient 2), the physiological start codon is skipped, thus predicting that the protein is not translated. We consulted the genomic databases Human Genome Segmental Database (http://projects.tcag.ca/humandup/), DECIPHER (http://decipher.sanger.ac.uk/), the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp) and the UCSC Genome
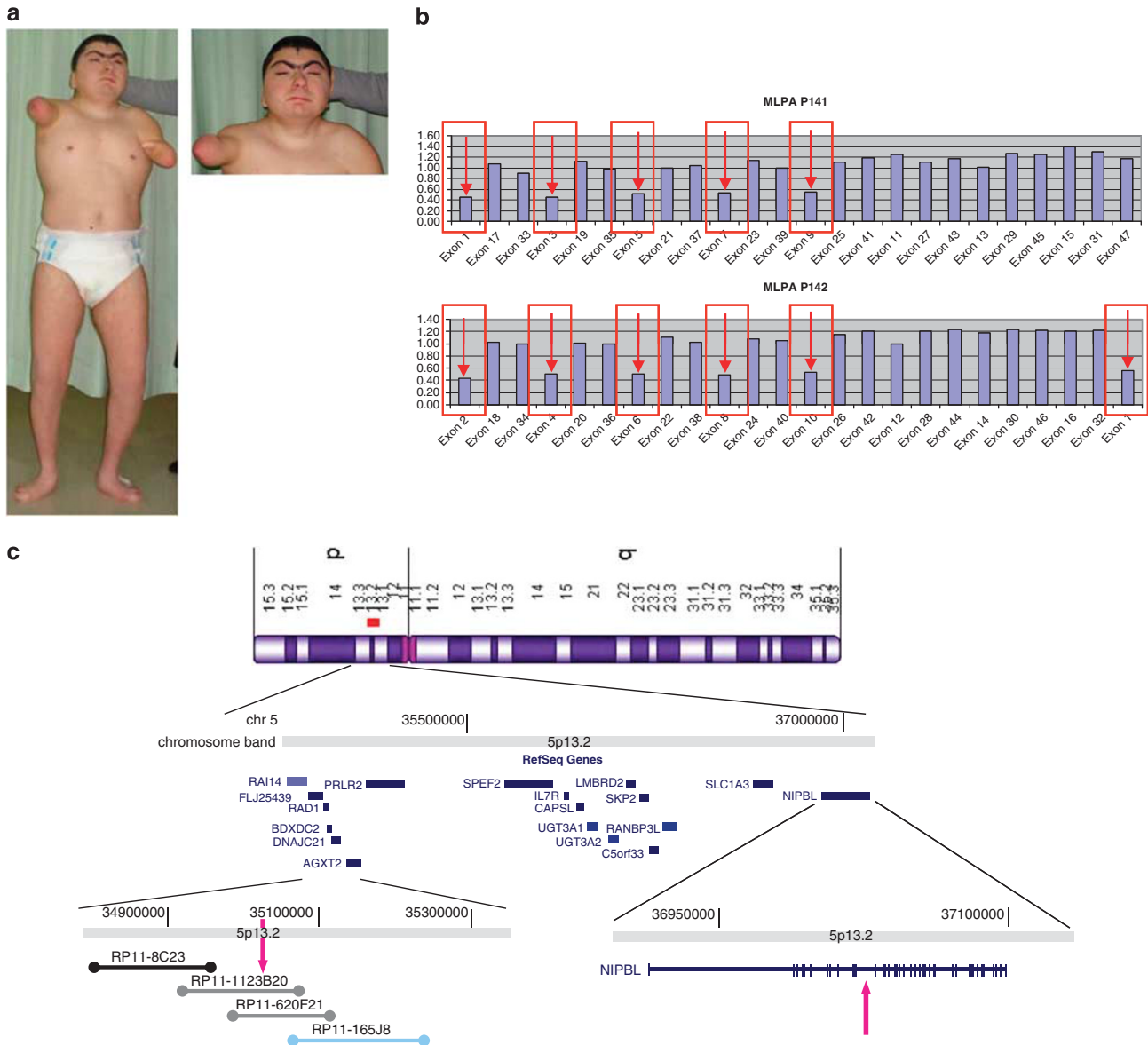
Figure 2 The clinical and genomic data of patient 1, suggesting a contiguous gene syndrome involving *NIPBL*. (**a**) Severe limb reduction and typical CdLS facial dysmorphism. (**b**) In the MLPA histograms, the red rectangles and arrows indicate the halved columns of the deleted exons: exon 1–10. (**c**) Ideogram of chromosome 5 and genomic details of the deleted 2 Mb region at 5p13.2 (red bar): the BAC clones used for the FISH characterisation are represented by horizontal bars and the *NIPBL* gene is schematically shown with all of its exons. MLPA indicated that the first 10 exons of *NIPBL* are deleted. The metaphase panels showed that RP11-165J8 gave only one signal, RP11-1123B20 and RP11-620F21 gave one normal and one faint signal, and RP11-8C23 gave two signals mapping outside the deleted region.

Browser (http://www.genome.ucsc.edu/), none of which includes any of the reported NIBPL rearrangements.

Clinical and molecular characterisation
All the clinical details of the seven patients are summarised in Table 1.

Patient 1
Patient 1 is a 36-year-old man with typical CdLS facial features (Figure 2a), pre- and post-natal growth delay, upper limb reduction, severe intellectual disability associated with the absence of speech, deafness, and gastro-esophageal reflux (GER). His clinical score of 38 is the most severe in our CdLS cohort. To investigate whether the

MLPA-assessed deletion of exons 1–10 (Figure 2b) extends upstream of the *NIPBL* gene, we mapped parent-to-proband segregation of the SNPs in the 5′UTR region (data not shown). Of the informative markers, rs10035888 (at 36384705) mapped within the deletion, whereas rs10036454 mapped outside it, as the proband was heterozygous. The telomeric breakpoint of the deletion was refined by means of FISH analysis. As shown in Figure 2c, the region targeted by probe RP11-165J8 is deleted, that targeted by BAC RP11-8C23 is not deleted, and RP11-1123B20 and RP11-620F21 give signals of decreased intensity on one chromosome 5p. Accordingly, the telomeric breakpoint of the deletion lies in the 30 kb region shared by clones RP11-1123B20 and RP11-620F21. The deletion was estimated to span 2 Mb,
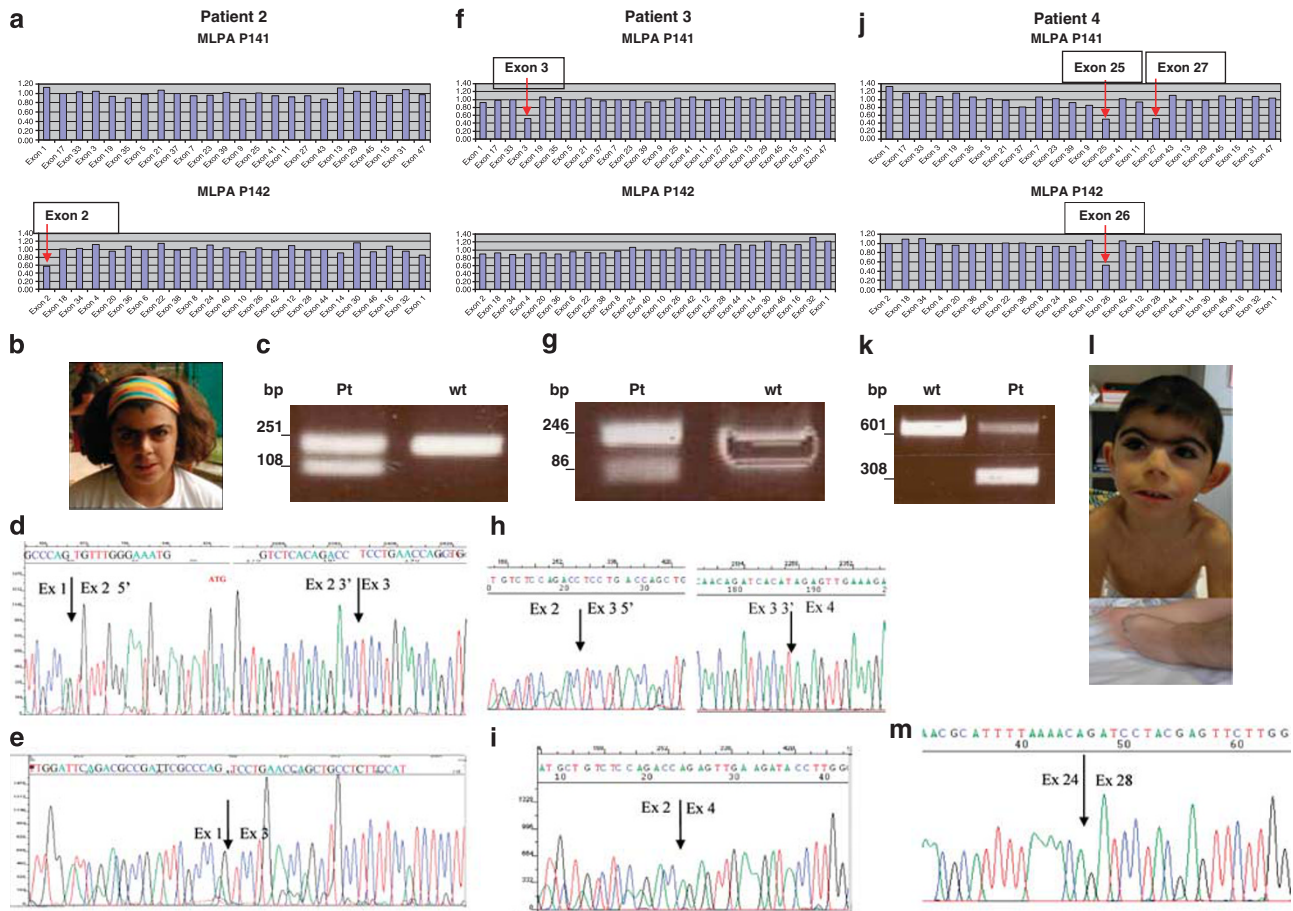
Figure 3 Vertical panels showing (from top to bottom) MLPA analysis (**a**, **f** and **j**), RT-PCR (**c**, **g** and **k**), and the sequencing of the wild type (**d** and **h**) and aberrant (**e**, **i** and **m**) transcripts of patients 2, 3, and 4. In the MLPA histograms, the red arrows indicate the halved columns of the deleted exons: exon 2 in patient 2, exon 3 in patient 3, and exons 25, 26, and 27 in patient 4. All of the RT-PCRs revealed a wild type and a lower band corresponding to the deleted fragment, whose sequence indicates the exact splice-junction. The pictures of patients 2 (**b**) and 4 (**l**) show the typical facial dysmorphism of CdL syndrome.

extending for about 1.9 Mb outside *NIPBL* to include the *AGXT2* (alanine–glyoxilate aminotransferase 2) gene at the telomeric end and other 12 genes, one of which is *SLC1A3* (Solute Carrier Family 1) that codes for the transporter molecules regulating neurotransmitter concentrations at the excitatory glutamatergic synapses of the mammalian central nervous system, and may contribute to the patient's severe behavioural disorder.

Patients 2 and 3

Patient 2 is a 16-year-old girl with facial dysmorphism (Figure 3b) obesity, mild GER, and moderate intellectual disability. Her clinical score was 20, in the mild range, as attested by the fact that she does not show any growth delay or limb reduction, and is also able to speak. MLPA revealed that the area of exon 2 is halved (arrowed in Figure 3a), and RT-PCR detected both wild-type and aberrant transcripts (Figure 3c). Sequencing showed that the size of the aberrant band was 108 bp, thus indicating the exact skipping of exon 2 (Figures 3d and e). The mutation (c.-75_+65del) leads to the removal of the ATG start codon. Patient 3 is a 3-year-old girl with mild facial dysmorphism, post-natal growth delay, mild to moderate psychomotor delay, absent speech and GER. MLPA detected a half-dose of exon 3 (arrowed in Figure 3f) corresponding to an aberrant

transcript (Figure 3g) that exactly skips exon 3 (Figures 3h and i). The mutation (c.65_230del) predicts a truncated pLeu22GlnfsX3 protein (Figure 1b).

Patient 4

A four-year-old boy with characteristic facial dysmorphism, pre- and post-natal growth delay, limb malformations (Figure 3l), severe intellectual disability and the absence of speech. His clinical score was 23. MLPA revealed the deletion of *NIPBL* exons 25, 26 and 27 (Figure 3j). Direct sequencing showed that the aberrant transcript detected by RT-PCR (Figure 3k) was due to the in-frame skipping of exons 25–27. The mutation (c.4921_5328del; Figure 3m) predicts a protein lacking 135 amino acids, p.Val1641_Gln1776del (Figure 1b).

Patients 5 and 6

The deletion of patient 5 and the duplication of patient 6 both affect *NIPBL* exon 32. Patient 5 is a 12-year-old boy with a clinical score of 34 (severe), classic CdLS dysmorphism (Supplementary Figure 1b), severe growth delay (also recorded prenatally), kidney and genital malformations, myopia, deafness and profound intellectual disability. MLPA revealed a reduction in the area of exon 32 (arrowed in Supplementary Figure 1a), but the mutation could not be evaluated
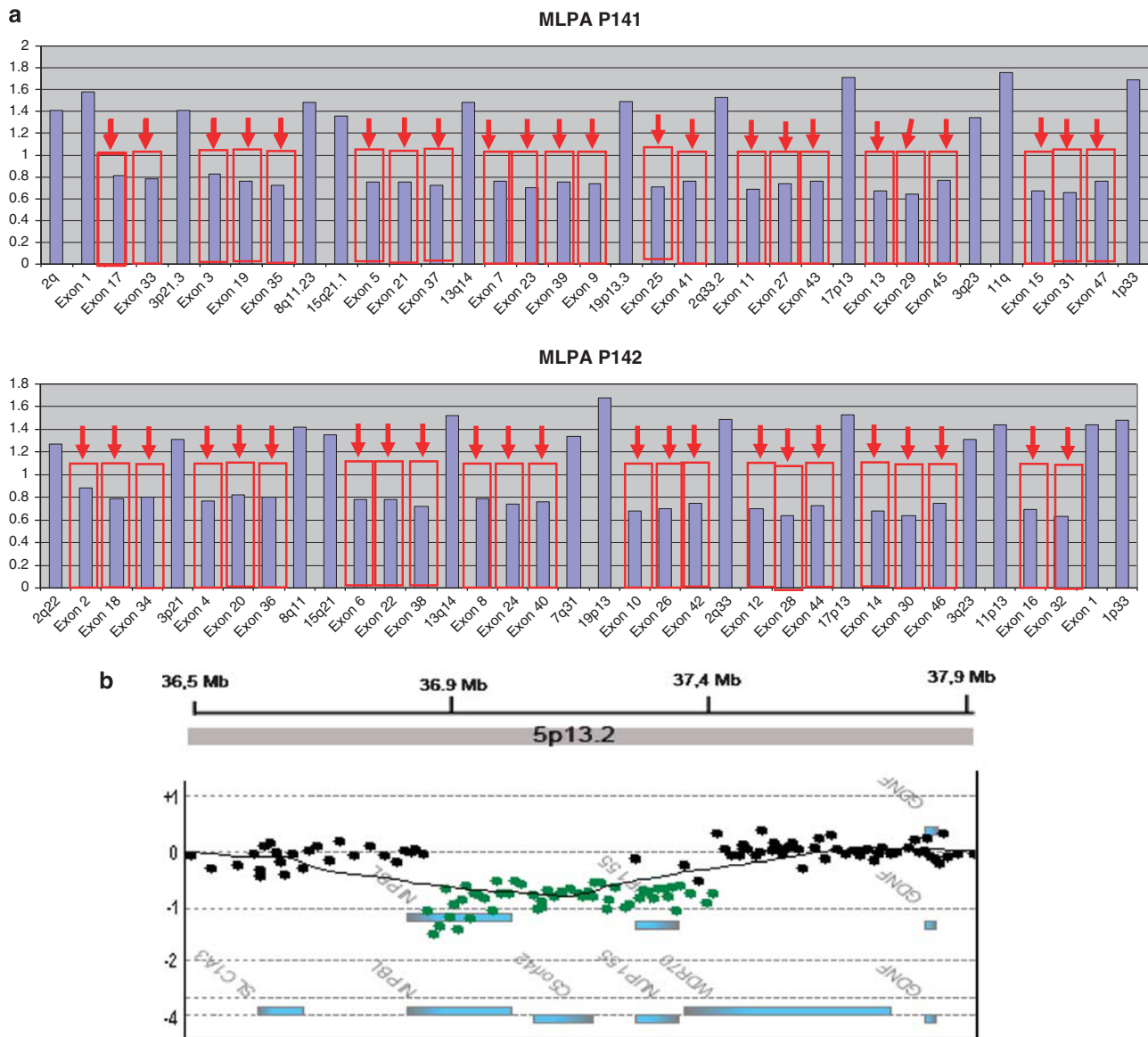
Figure 4 Clinical and genomic data of patient 7. (a) MLPA histograms: NIPBL exons arrowed in red (2–47) show reduced height in comparison with the control probes (0.7 *vs* 1.4). (b) a-CGH profile focussing on a 1.4 Mb window within the 5p13.2 interval containing the *NIPBL* deletion; the scatter plot shows a deletion in 5p13.2 (the shift is marked by green dots under 0). Each dot represents a single probe.

at transcript level. In patient 6, MLPA revealed a double dose of exon 32 (Supplementary Figure 1c). This patient was sent to our centre from South America, which is why we could not collect any clinical information suitable for assigning an overall score. Genomic q-Real time PCR confirmed the presence of a double amount of PCR product corresponding to exon 32 duplication (Supplementary Figure 1d). Amplification of this exon using exon 32 flanking primers did not reveal any longer PCR product, thus making it likely that the duplication breakpoints map within introns 31 and 33, and involves part of their sequence.

Patient 7
Patient 7 is a girl who has been suspected of having CdLS since she was 3 months old; a detailed clinical evaluation at the age of 6 years indicated a global score of 26. She has typical facial dysmorphism, full-body hirsutism, clinodactyly of the left little finger and limited elbow movement, absent the ring and little fingers of her right hand,

gastroesophageal reflux, mild hypotrophy of the corpus callosum, and myopia with moderate growth retardation, psychomotor delay and intellectual disability. MLPA revealed control probes with values of about 1.4 and almost all of the *NIPBL* exons with values of about 0.7, thus suggesting a deletion from exon 2 to the last exon of the gene (Figure 4a). The MLPA data were validated by means of a-CGH, which revealed a large deletion (515 kb, from kb 36 950 899 to 37 466 333) involving the whole *NIPBL* gene and extending to within the 3′UTR region (chromosome 5p13.3) (Figure 4c). The telomeric deletion breakpoint maps within an 8.7 kb region between 36 942 193 bp and 36 950 899 bp, whereas the centromeric breakpoint is located within an 8.9 kb interval between 37 466 333 bp and 37 475 302 bp. The deleted region includes most of the coding sequence of *NIPBL* (from exon 2 to the 3′UTR) plus *C5orf42* (chromosome 5 open reading frame 42 for a putative transmembrane protein), *NUP155* (coding for a nucleoporin protein) and part of the *WDR70* gene containing a domain homologues to *WDR40*. The clinical

presentations of the CdLS patients whose *NIPBL* rearrangements were revealed by MLPA are very heterogenous, with clinical scores ranging from the severe scores of patients 1 and 5 (38 and 32) to the moderate scores of patients 4 and 7 (23 and 26) and the very mild scores of patients 2 and 3 (20 and 18). Only the carriers of large deletions (1 and 7) show limb reduction or anomalies, whereas all of the patients except patient 3 who has a normal growth curve are characterised by mild-severe intellectual disability, typical facies and growth retardation. Patient 6 is not included in this comparative overview because of the lack of clinical details.

DISCUSSION

A comprehensive *NIPBL* locus-specific mutation database has recently been set up using LOVD (Oliveira *et al*,[25] http://www.lovd.nl/*NIPBL*), which among the different mutation types identified in 196 CdLS patients, assigns decreasing frequencies to small deletions (27.6%), missense (21.1%), nonsense (17.3%) and splicing mutations (17.1%), and small duplications (13.6%). There are few insertions and indels (1.5% each), and gross deletions/duplications are limited to the two described by Bhuiyan *et al*[12] and Vrouwe *et al*.[11] Our cohort of 200 investigated CdLS patients shows a mutation distribution that is quite similar to that of the LOVD database, except for the higher frequency of splicing defects (33.8%). Whenever we could investigate the effects of splice mutations, we have always found that the causative lesion was the lack of a whole exon (data not shown). This was the rationale for the present study as it favoured the view that whole-exon deletions with effects similar to splicing defects might be responsible for CdLS in a subset of patients. The use of MLPA to test samples from a group of 132 CdLS patients who were negative to the standard *NIPBL* test validated this hypothesis by revealing four intragenic single- or multi-exon deletions and one single-exon duplication. MLPA also identified two unusually large *NIPBL* deletions.

Interestingly, the extent and gene content of the deletion carried by patient 1 allows to classify him as a first reported carrier of a contiguous gene syndrome, including *NIPBL*. Two instances of deletions of chromosome 5p13 involving *NIPBL* have been reported in CdLS patients,[17,18] both of which were cytogenetically detectable and occurred in infants who died perinatally, thus preventing a full description of their clinical presentation and the precise mapping of the deletion breakpoints. Three large *NIPBL* imbalances (all detected by means of MLPA) have subsequently been reported,[11–13] two of which are cited in the LOVD *NIPBL*-specific database, but all appear to be intragenic. On the basis of these data and those of the present study, it seems that, although rare, large rearrangements affecting *NIPBL* may occur, as was predicted by the results of a study based on a 'reverse genomics approach' that did not focus on CdLS patients.[16] This study, which had the aim of establishing the dose effect of cohesin-associated genes by means of targeted oligonucleotide a-CGH analyses, found five patients carrying duplications on the chromosome 5 region containing *NIPBL*, four of which extended beyond the *NIPBL* gene.[16] Our study shows that large *NIPBL* deletions can also be detected by means of MLPA. The large multigenic deletion of patient 1 could be correlated to his extremely severe phenotype. This *de novo* deletion spans about 2 Mb from the centromeric breakpoint within *NIPBL* to the telomeric breakpoint (13 genes), as indicated by the complementary use of MLPA, FISH and SNP/microsatellite analyses. Physical examination showed the presence of all of the signs of CdLS, including pre- and post-natal growth retardation, typical facial dysmorphism (Figure 2a), delayed psychomotor development, severe intellectual disability, bilateral severe upper limb reduction (Figure 2a), small feet, deafness and gastroesophageal reflux. This

severe presentation is probably due to the combined haploinsufficiency of dose-sensitive genes located in the deleted region (Figure 2b). One relevant gene may be *SLC1A3*, which encodes a member of a family of high-affinity sodium-dependent transporter molecules that regulate neurotransmitter concentrations at the excitatory glutamatergic synapses of the mammalian CNS.[19] Glutamate and aspartate are excitatory neurotransmitters involved in various CNS diseases. Heterozygous mutations in the *SLC1A3* gene have been reported in one sporadic case and in three members of a family affected by type 6 episodic ataxia,[20,21] and *SLCA3* was included in the duplication of one of the five patients with intellectual disability, developmental delay and severe behavioural abnormalities described by Yan *et al*.[16] We hypothesise that *SLC1A3* may contribute to the neurological impairment of our patient 1 (which is extreme even among CdLS patients), and minimise the role of the other genes. Interpreting the effect of the loss of a single gene mapping to a deleted region is very difficult, especially when the deletion is too large to identify the specific contribution of each gene to the phenotype. Nevertheless, as the patient has all of the signs of CdLS without any definitive extra-CdLS signs, it seems that *NIPBL* is the major dose-sensitive gene. Despite its more limited extension, the deletion of patient 7 is similar insofar as it involves the whole of *NIPBL* gene and 3′UTR regions that contain ORFs whose functional roles are still unclear, the *NUP155* gene, and part of the interrupted *WDR70* gene encoding for a WD repeat containing protein 70, which is found in a number of eukaryotic proteins with a wide variety of functions, including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly, and typically contains a GH dipeptide 11–24 residues. The proband has a classic CdLS phenotype with a moderate/severe score and partial limb reduction, all of which are signs attributable to the absence of *NIPBL*. Interestingly, three of the five patients described by Yan *et al*[16] and carrying *NIPBL* duplications also included these genes, and one of them had short fifth fingers. The centromeric breakpoint of our patient 7 falls between bp 37 466 333 and bp 37 475 302, very close to the centromeric breakpoint of the reported duplications, which map between 37 083 320 and 37 420 236. We hypothesise that both reciprocal rearrangements may affect the function of the *NUP155* gene, although the contribution to the clinical phenotype of our patient is difficult to define. Interestingly, the closeness of the centromeric breakpoint of our patient to those reported by Yan *et al*[16] suggests that these rearrangements may be mediated by common genomic mechanisms primed by DNA sequence motifs.

As a whole, our findings indicate that MLPA can make a substantial contribution to defining the entire mutational repertoire of *NIPBL* featuring as unique approach in detecting *NIPBL* intragenic single or multi-exon deletions and duplications that might be overlooked by a-CGH, as exemplified by patients 2, 3, 5 and 6 who would have never been disclosed by a-CGH. The intragenic exon 3 deletion of patient 3 (which cDNA analysis indicates as predicting protein truncation) deserves comment because this exon encodes a delangin domain that interacts with the Mau-2 protein required for the binding of cohesin to chromosomes.[22] The consequence of the deletion (p.Leu22GlnfsX3) is the same as that predicted by the nonsense mutation described in the literature[23,24] and the splicing mutation (c.65-5A.G) found in two of our CdLS patients[7] (plus unpublished data). Patients 2 and 3, whose deletions skip exons within the *NIPBL* amino terminus, have a very mild phenotype (clinical scores of 20 and 18) even though it is predicted that they are haploinsufficient for the protein. Whereas patient 4, who has an in-frame deletion damaging the HEAT domain, has a severe phenotype. It may be that the absence of a truncated protein is less damaging than the expression of an

aberrantly truncated protein or that some other factors interact to modulate the complex CdLS phenotype. Unlike the Oliveira database, in which exon 32 does not seem to harbour any mutations in CdLS probands,[25] MLPA revealed two rearrangements involving exon 32 in our cohort (one deletion and one duplication), thus showing its usefulness in delineating the *NIPBL* mutational spectrum. Although exon 32-encoded protein includes 54 amino acids, we could not establish the in-frame occurrence of the deletion by means of transcript analysis, but the severe phenotype of patient 5 can be explained by the inclusion of the HEAT domain in the aberrant protein. The use of MLPA analysis after the standard *NIPBL* test identified a further 5.3% of genetic lesions accounting for CdLS in *NIPBL*-negative patients, including seven deletions (four intragenic and two extending beyond the *NIPBL* gene), and one duplication. When applied to CdLS patients *per se*, MLPA is capable of detecting the molecular lesion in 3.5% (7/200) of the patients. For these reasons, we strongly encourage the inclusion of MLPA as an obligatory step in *NIPBL* test. We have applied MLPA to a cohort of CdLS patients, negative to both *NIPBL* and *SMC1A* genes, which has been cumulated across time. However, according to the gained experience, we believe that the diagnostic flow chart of patients with a consistent clinical diagnosis of CdLS should start with the standard test of the major *NIPBL* gene, be then completed by *NIPBL* MLPA test and continued by the screening of *SMC1A* gene. The a-CGH approach overlaps MLPA in detecting *NIPBL* large rearrangements, but is less sensitive than MLPA in detecting small intragenic losses/gains. Conversely, a-CGH can be pursued especially in patients with a CdLS-like clinical presentation, being suitable to detect imbalances of genomic regions other than those harbouring the currently known *CdLS* genes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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