ORIGINAL ARTICLE

High rate of mosaicism in individuals with Cornelia de Lange syndrome

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ABSTRACT

Background Cornelia de Lange syndrome (CdLS) is a well known malformation syndrome for which five causative genes are known, accounting for ~55–65% of cases. In this study, we hypothesised that mosaicism might explain some of the ~35–45% of cases without detectable mutation in DNA derived from lymphocytes; we investigated the frequency of *NIPBL* mutations in buccal cells in individuals negative for mutations in any of the five genes in lymphocytes; and we evaluated the efficiency of obtaining DNA from buccal swabs and the best strategy for optimal mutation detection in CdLS.

Methods Buccal swabs were obtained from eight mutation positive and 13 mutation negative individuals with clinically diagnosed CdLS, following informed consent. We then forwarded instructions and a single mouth swab to the families; if subsequently insufficient DNA was obtained, we re-sent two mouth swabs. Buccal cells were screened for *NIPBL* mutations using Sanger sequencing techniques.

Results Sufficient DNA for analysis was obtained in 21/22 individuals. In all six tested individuals with a known *NIPBL* mutation and in two with a known *SMC1A* mutation, the mutation was confirmed in buccal cells. In 10 of the 13 tested individuals without detectable mutation in lymphocytes a *NIPBL* mutation could be detected in buccal cells. Clinically there were no significant differences between patients with a germline and mosaic *NIPBL* mutation.

Conclusions Somatic mosaicism for an *NIPBL* mutation is frequent (10/44; 23%) clinically in reliably diagnosed CdLS individuals. Obtaining buccal swabs at the time a blood sample is obtained will facilitate adequate molecular analysis of clinically diagnosed CdLS patients.

INTRODUCTION

Cornelia de Lange syndrome (CdLS, or Brachmann-de Lange syndrome; OMIM 122470, 300590, and 610759) is a well known malformation syndrome characterised by a distinctive face, prenatal and postnatal growth retardation, limb malformations, and intellectual disability. To date, five causative genes have been identified: *NIPBL*, *SMC1A*, *SMC3*, *RAD21*, and *HDAC8*.[1–6] Each one of these genes has a function in the sister chromatid cohesion process and CdLS is therefore termed as a cohesinopathy.[7] Mutations in *NIPBL* are found in ~50–60% of cases; the other genes account together for about 5% of clinically confirmed diagnoses, indicating that up until now CdLS can only be molecularly confirmed in ~55–65% of patients (table 1).[1 8–23] Studies by us and others using whole exome sequencing techniques failed to show pathogenic variants in CdLS individuals in whom mutations in the five known pathogenic CdLS genes had already been excluded (unpublished data). We hypothesised that this was caused by mosaicism and that searching for mutations using other tissues might yield additional mutations in genes known to cause CdLS.

We describe here the results of *NIPBL* mutation analysis in buccal cells in CdLS individuals without a detectable mutation in one of the five known genes in lymphocytes. Furthermore, we report on the efficiency of molecular analysis of buccal swabs, genotype–phenotype correlations in patients with and without mosaicisms, and discuss strategies for optimal mutation detection in CdLS.

METHODS

Recruitment

In our earlier study[9] we studied 39 CdLS individuals, to which we added five other CdLS individuals who were negative for *NIPBL* mutation analysis in lymphocytes. We asked eight mutation positive CdLS individuals described in the earlier study[9] to participate by obtaining a buccal swab, to test for the reliability of molecular analysis of buccal swabs. All agreed. We then asked 17 individuals in whom no mutation was found in the five known genes to participate. Fourteen of them responded with consent.

We forwarded a single mouth swab to all families, asking parents to perform a buccal swab of their child. If insufficient DNA was obtained, we re-sent two mouth swabs to the families and asked them to repeat the procedure. No particular modifications were applied to increase the isolation of DNA from the swabs.

Severity scores

The severity score[9] of the CdLS individuals in the earlier study was updated and the same severity score was added in the five patients who were not in the earlier study.

Molecular investigations

Genomic DNA was isolated from buccal swabs by using the Maxwell Buccal Swab LEV DNA Purification kit (Promega). Primers used for amplification of the 46 *NIPBL* coding exons (exons 2–47, NM_133433.3) and the corresponding exon–intron boundaries were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). PCR fragments were sequenced using the Big Dye Terminator cycle sequencing kit v2 (Applied Biosystems), and

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Table 1 Overview of studies describing results of mutation analysis in four or more individuals with clinically diagnosed CdLS*

Author	Number of patients	Methods	Mutations							
			NIPBL N (%)	SMC1A N (%)	SMC3 N (%)	RAD21 N (%)	HDAC8 N (%)	Mosaicism N (%)	Any mutation N (%)	No detectable mutations N (%)
Gillis *et al* (2004),	120	Sequencing/FISH	56 (47%)						56 (47%)	64 (53%)
Deardorff *et al* (2007),	115 NIPBL-	Sequencing		10 (9%)	1 (1%)‡				11 (10%)	104 (90%)
Chatfield *et al* (2012)	319†		130 (41%)	15 (4.7%)	1 (0.3%)				146 (46%)	173 (54%)
Borck *et al* (2004, 2006, 2007)	30	Sequencing/ aCGH/sequencing 5′ÚTR	13 (43%)	2 (7%)	0				15 (50%)	15 (50%)
Miyake *et al* (2005)	15	Sequencing/FISH	4 (27%)						4 (27%)	11 (73%)
Yan *et al* (2006),	28	Sequencing	13 (46%)						13 (46%)	15 (54%)
Ratajska *et al* (2010)	11 (NIPBL-/ SMC1-	MLPA, aCGH	1 (9%)§	0					1 (9%)	10 (91%)
Selicorni *et al* (2007), Gervasini *et al* (2008), Russo *et al* (2012)	200	Sequencing/FISH aCGH/MLPA	75 (38%)	0	0				75 (38%)	125 (62%)
Schoumans *et al* (2007)	a: 11	a: Sequencing	a: 7 (64%)	0					a: 7 (64%)	a: 4 (36%)
	b: 4	b: MPLA/5′UTR/ aCGH	b: 1 (25%)¶						b: 1 (25%)	b: 3 (75%)
Pie *et al* (2010)	30	Sequencing	11 (37%)	3 (10%)	0				14 (47%)	16 (53%)
Zhong *et al* (2012)	4	Sequencing	2 (50%)	0	0				2 (50%)	2 (50%)
Bhuiyan *et al* (2006, 2007, present study)	44	Sequencing/ MLPA/sequencing buccal	25 (57%)**	2 (5%)	0	0	0	10 (23%)††	37 (84%)	7 (16%)

*If a gene was not sequenced in the study the square is left blank.
†Individuals with congenital heart disease.
‡1/96 studied.
§Deletions detected by MLPA.
¶9p duplication.
**One with deletion detected by MLPA.
††Until now in 4/17 without detectable mutation in lymphocytes no buccal swabs could be obtained.
aCGH, array based comparative genomic hybridisation; CdLS, Cornelia de Lange syndrome; FISH, fluorescence in situ hybridisation; MLPA, multiplex ligation dependent probe amplification.

analysed on a 3130 Genetic Analyser sequencing machine (Applied Biosystems). Sanger sequencing does not yield reliable quantitative results. The ratio between the variant and wild-type of a locus was evaluated by eyeballing only.

Ethics

The present study is part of a wider study in individuals with CdLS ('CoDeLaGe') and has been approved by the medical ethics committee of the Academic Medical Center in Amsterdam, and by the board of the Dutch CdLS support group.

Statistics

For analysis of correlations between ordinal categorical variables, the χ^2 test for trend was used. Analysis was performed using SPSS V.20. The significance threshold was set at $p<0.05$.

RESULTS

We obtained buccal swabs from a total of 22 individuals with CdLS and eventually sufficient DNA for mutation analysis could be harvested from 21/22. In five individuals we needed an extra pair of buccal swabs as the amount of DNA obtained from the first swab was insufficient. In one patient in whom we had found no mutation in lymphocytes, sufficient DNA could not be harvested from buccal cells despite collection of an extra set of buccal swabs.

In the total group of 44 individuals with CdLS we found 25 mutations in *NIPBL*, two in *SMC1A*, and none in the three other genes (*SMC3*, *RAD21*, *HDAC8*) (table 1). We were able to confirm in DNA derived from buccal cells the mutation found in *NIPBL* in all six individuals in whom such a mutation was earlier detected in DNA derived from lymphocytes (table 2); also the *SMC1A* mutation was retrieved in DNA isolated from buccal cells in the two tested CdLS individuals. Of 13 individuals with CdLS in whom no mutation was detectable earlier in lymphocytes, a mutation in *NIPBL* was found in buccal swabs in 10 of them (tables 2 and 3). The ratio between the pathogenic variant and wild-type was estimated to be about equal. As this mosaicism was unexpectedly high and might in theory point to an increase for *NIPBL* mutations in buccal cells irrespective of the presence of CdLS, we obtained a mouth swab from three healthy controls and excluded *NIPBL* mutations in them. Also the two CdLS individuals with an *SMC1A* mutation in lymphocytes were checked for an *NIPBL* mutation in buccal cells and were found to be negative.

Table 2 Mutation detection rate in buccal swabs in relation to findings in lymphocytes

	NIPBL mutation in buccal cells detected	*NIPBL* mutation in buccal cells not detected	Total
NIPBL mutation in lymphocytes detected	6	0	6
NIPBL mutation in lymphocytes not detected	10	3	13
Total	16	3	19

Table 3 Mosaic *NIPBL* mutations detected in present CdLS cohort

	Lymphocytes	Buccal cells	Comment
1	Wild type	c.358+3G>T	1, 2
2	Wild type	c.4543G>T, p.Glu1515*	
3	Wild type	c.1345C>T, p.Gln449*	
4	Wild type	c.2389C>T, p.Arg797*	1
5	Wild type	c.7263+5G>A	2
6	Wild type	c.742_745dup, p.His 249Profs*9	
7	Wild type	c.7168G>A, p.Ala2390Thr	1
8	Wild type	c.790del, p.Met264*	
9	Wild type	c.3327del, p.Asp1110Metfs*63	
10	Wild type	c.459-9G>A	2
11	Wild type	Wild type	
12	Wild type	Wild type	
13	Wild type	Wild type	
14	c.2479_2480del, p.Arg827Glyfs*2	c.2479_2480del, p.Arg827Glyfs*2	1
15	c.2771del, p. Asn924Thrfs*5	c.2771del, p.Asn924Thrfs*5	
16	c.6156G>C, p.Glu2052Asp	c.6156G>C, p.Glu2052Asp	
17	c.2324A>G, p.Lys775Arg	c.2324A>G, p.Lys775Arg	
18	c.7062+1G>A	c.7062+1G>A	2
19	c.6892C>T, p.Arg2298Cys	c.6892C>T, p.Arg2298Cys	1

1, Detected in other CdLS patients as well (LOVD database); 2, In silico splicing predictions show disrupted splice sites (Alamut prediction).
CdLS, Cornelia de Lange syndrome.

We checked in DNA isolated from lymphocytes in each CdLS individual whether the variant detected in their buccal cells was present in the lymphocytes as well, by re-sequencing (Sanger sequencing) for that particular mutation, but none was retrieved (figure 1). In three CdLS individuals from the original group previously reported,[9] no mutation was detected in either lymphocytes or buccal swabs.

The clinical characteristics of the CdLS individuals with a mutation detectable in lymphocytes, those with an *NIPBL* mutation detectable only in buccal swabs, and those without detectable mutation were compared using the severity score (table 4). The comparison is limited to the 37 CdLS individuals for whom we had sufficient data. Statistical analysis failed to show any significant difference between the group of individuals with a germline *NIPBL* mutation and mosaicism for an *NIPBL* mutation, and between the group of individuals with a germline *NIPBL* nonsense mutation and a mosaicism for an *NIPBL* nonsense mutation ($p=0.704$ and $p=0.335$, respectively). However, numbers were small and minor differences may have gone unrecognised.

DISCUSSION

Molecular confirmation of the clinical diagnosis of CdLS is of the utmost importance for adequate genetic counselling of families, and is critical in exploring genotype–phenotype correlations and for understanding the pathogenesis of the various manifestations of CdLS. We report here an unexpected and unusually high frequency of somatic mosaicism in CdLS individuals.

Mosaicism in CdLS has been reported before only infrequently: a chromosomal mosaicism was reported in 1965,[24] and

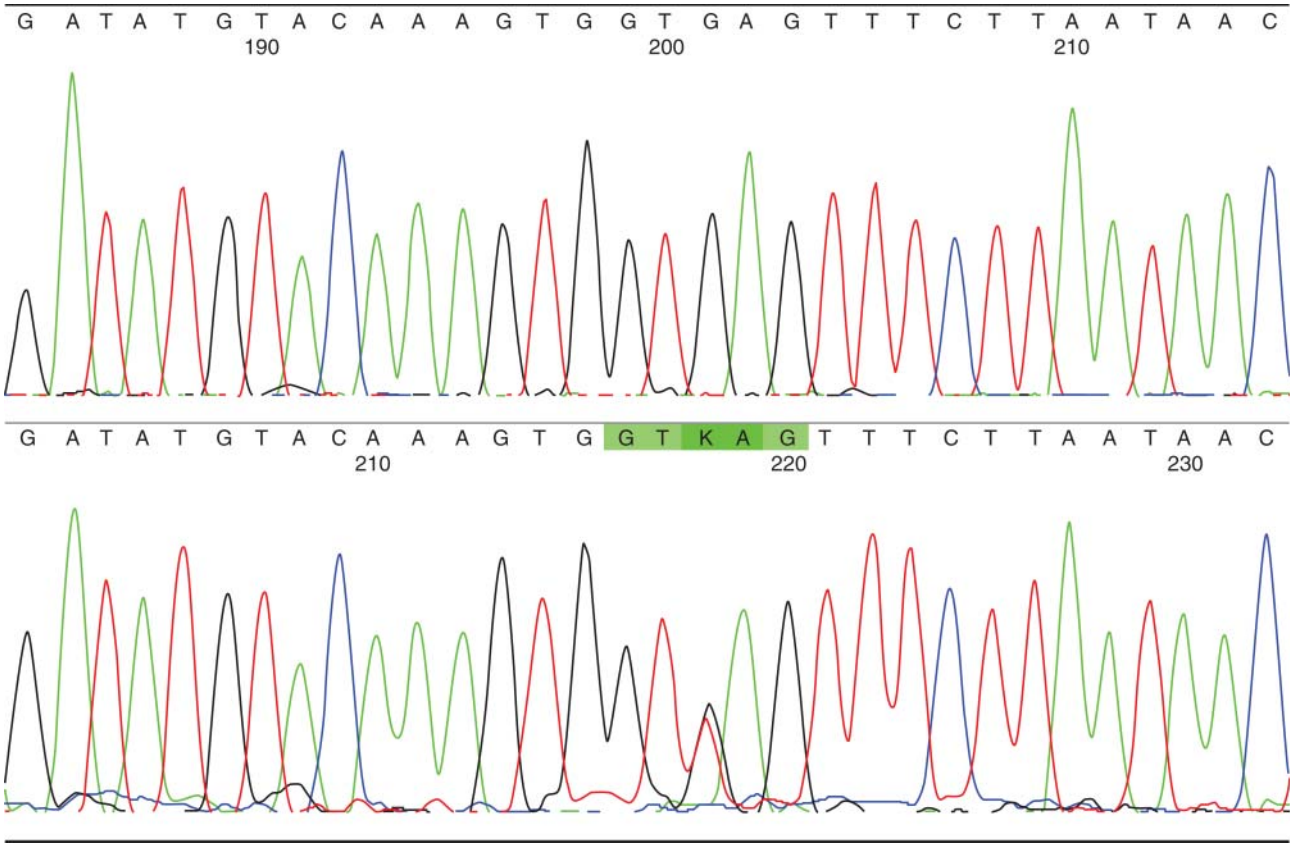

Figure 1 Chromatogram showing the mutation c.358+3G>T in intron 4 identified in buccal DNA (lower lane) which is not present in lymphocyte DNA (upper lane).

Table 4 Severity score features related to molecular findings

	Molecular findings				
	***NIPBL* mutation+in lymphocytes**		***NIPBL* mutation+mosaic**		***NIPBL* mutation—in lymphocytes and buccal cells**
Number of patients	25		10		2
Gender (M/F)	14/11		3/7		0/2
Age median (min–max) (year)	23.5 (10.5–54.2)		15.2 (3.8–33)		22.1 (13.5–31)
Birth weight mean (SD)/ median (g)	2227 (703) / 2110		2325 (506) / 2375		3395 (1266) / 3395
Postnatal growth*					
>P75	4 (17)		3 (30)		2 (100)
P25–P75	15 (62)		7 (70)		
<P25	5 (21)				
Skull growth*					
>−2SD	3 (14)		2 (20)		1 (50)
<−2SD and >−4SD	11 (50)		4 (40)		1 (50)
<−4SD	8 (36)		4 (40)		
Limbs*					
No reduction defect	19 (79)		10 (100)		2 (100)
Partial reduction defect	1 (4)				
Severe reduction defect	4 (17)				
Face					
Classic type	20 (80)		7 (70)		
Mild type	5 (20)		2 (20)		
Possible CdLS			1 (10)		2 (100)
IQ score					
0–20	8 (32)		2 (20)		
21–35	8 (32)		4 (40)		1 (50)
36–50	5 (20)		3 (30)		
51–70	4 (16)		1 (10)		
71–85					1 (50)
Total severity score*	Nonsense mutation	Missense mutation	Nonsense mutation	Missense mutation	
Classic type	12 (86)	3 (50)	5 (56)		0
Mild type	1 (7)	1 (17)	4 (44)	1 (100)	1
Possible CdLS	1 (7)	2 (33)			1

Data are displayed as n (%) unless stated otherwise.
*Reliable data on some patients missing.
CdLS, Cornelia de Lange syndrome.

in 2010 a report of mosaicism for a c.2827delA mutation in *NIPBL* was published.[25] The cohort of individuals with CdLS investigated in this study has been previously reported in an earlier genotype–phenotype study[9] and a selection bias seems unlikely.

A similarly high frequency of mosaicism in a malformation syndrome with or without intellectual disability is unknown to us, except for entities that already show clear signs fitting mosaicism, such as asymmetries or pigmentation abnormalities.[26–28] In the present series of people with CdLS a single individual showed a difference in colour between the left and right eye (figure 2), but otherwise none showed a significant clue for mosaicism. Heterochromia of the iris occurs in non-mosaic Mendelian conditions such as Waardenburg syndrome, but it is not a recognised sign in CdLS and must be very unusual as reports on many individuals with CdLS have been published. Heterochromia of the iris can occur in disorders caused by mosaic mutations such as Proteus syndrome, and therefore it seems possible that the heterochromia found in an individual mosaic for an *NIPBL* mutation is associated with the mosaicism. We cannot exclude, however, that its presence is coincidental.

There are several other malformation syndromes with intellectual disability, such as Rubinstein-Taybi syndrome and Kabuki syndrome, in which molecular confirmation of the clinical diagnosis is possible in only a limited percentage of cases, and we suggest performing similar studies in these entities. We have used only buccal swabs as second tissue to evaluate, purposes, but it has to be determined in each entity whether this is the right tissue to use. It might be that other easily available tissues such as bladder epithelial cells and hair bulbs are more suitable in other disorders. We do not exclude the possibility that further mosaicism can be detected in CdLS if other tissues are studied as well. Screening for mosaicism is especially important before initiating next generation sequencing studies (NGS) to detect additional pathogenic genes. If settings in evaluating whole exome sequencing studies are adequately set, one may be able to detect very low levels of mosaicism in NGS, but this would be an expensive approach.

The high rate of mosaicism for *NIPBL* mutations detected in the present study is remarkable and remains as yet unexplained. Theoretically the main mechanisms underlying this include somatic mutations (shortly) after fertilisation, loss of mutations in lymphocytes due to reversion, and selection against mutant cells specifically in lymphocytes.[29] The absence of a difference in phenotype between CdLS individuals with a mosaic and germline *NIPBL* mutation argues against a somatic mutation

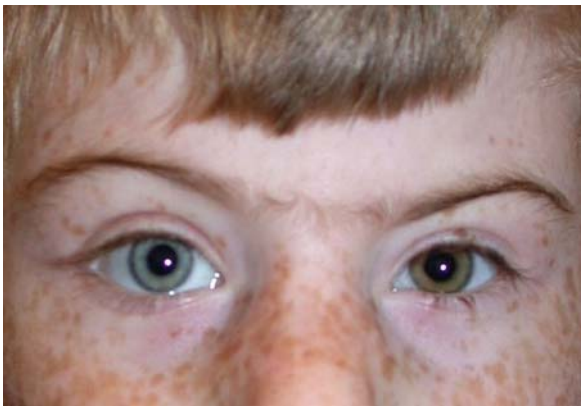

Figure 2 Individual with Cornelia de Lange syndrome and a mosaic *NIPBL* mutation showing differently coloured irides.

after fertilisation. Reversion is a rarely detected phenomenon and mainly known with skin disorders, and would be unusually frequent for the various *NIPBL* mutations detected in the present study. We favour the hypothesis that there is a selection against lymphocytes with the mutation. This selection should take place specifically in lymphocytes and not in other easily available tissues. One may speculate an external influence such as acetylation of the cohesion complex to be of significance here.

Buccal swabs were shown to be an adequate way to obtain DNA from a second tissue in the present study. Swabs are cheap, can be performed at home by parents or other caregivers, and the success rates in obtaining sufficient DNA after one (14/20) or a repeat swab (5/6) or both (19/20; 95%) were high, despite the fact that *NIPBL* is a relatively large gene. The families did not consider taking one or two buccal swabs to be a significant burden.

The detection of a somatic mutation in a significant number of individuals (10/44; 23%) allowed us to detect a causative mutation in 37/44 individuals (84%), which is high compared to earlier reported studies (table 1). Possibly in these studies a significant number of cases had somatic mosaicisms as well. There is no significant difference in the classical CdLS signs and symptoms between individuals with a causative mutation detectable in lymphocytes and those with a mutation detectable in buccal cells (table 4), and it seems impossible to discern in advance those with and without somatic mosaicism. We restricted the present molecular analysis in buccal cells to sequencing of only *NIPBL* as it is by far the most frequently mutated gene in CdLS. We plan to perform further analysis in DNA derived from buccal cells for the other four genes known to cause CdLS as well. The first results of Sanger sequencing of *SMC1A* of DNA isolated from buccal cells of two CdLS individuals who were negative for the five known genes in lymphocytes and for *NIPBL* in buccal cells indicated no mutation was present. Further analysis is in progress.

An efficient and effective screening strategy to detect mutations in individuals with clinically diagnosed CdLS is important in daily patient care. We have adapted our diagnostic strategy and take a pair of buccal swabs in each CdLS individual together with the initial blood sampling. We sequence the buccal sample first for a *NIPBL* mutation; if negative we continue by sequencing the other four CdLS candidate genes in lymphocytes. If an *NIPBL* mutation is identified on buccal swab DNA, we then sequence *NIPBL* in DNA isolated from lymphocytes, because finding the mutation in both tissues will have consequences for the recurrence risk. We anticipate that with time NGS techniques will be used in diagnostics, using a targeted analysis of the results for variants in the five genes known to cause CdLS. Despite the high sensitivity of this technique to detect mosaicisms we have sincere doubts as to whether it will allow detection of mosaic *NIPBL* mutations in CdLS individuals. NGS of DNA isolated from buccal cells is in principle possible but technically demanding and unlikely to be available for patient care in the near future. Individuals who will be negative for both lymphocyte and buccal cell studies will be candidates for NGS using samples of both parents as well (trio strategy).

CONCLUSION

We conclude that there is a significant number of CdLS individuals who have somatic mosaicism for an *NIPBL* mutation. DNA derived from buccal cells using a buccal swab is a reliable way to investigate whether a patient may have a somatic mosaicism if lymphocyte analysis has failed to show a mutation. Obtaining a buccal swab at the time the initial blood sample is obtained will facilitate adequate molecular analysis of clinically diagnosed CdLS individuals.

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