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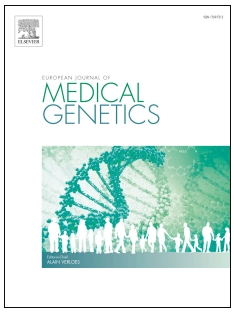

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Novel mosaic variants in two patients with Cornelia de Lange syndrome

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Abstract

Cornelia de Lange syndrome (CdLS) is a dominantly inherited developmental disorder caused by mutations in genes that encode for either structural (*SMC1A*, *SMC3*, *RAD21*) or regulatory (*NIPBL*, *HDAC8*) subunits of the cohesin complex. *NIPBL* represents the major gene of the syndrome and heterozygous mutations can be identified in more than 65% of patients. Interestingly, large portions of these variants were described as somatic mosaicism and often escape standard molecular diagnostics using lymphocyte DNA.

Here we discuss the role of somatic mosaicism in CdLS and describe two additional patients with *NIPBL* mosaicism detected by targeted gene panel or exome sequencing. In order to verify the next generation sequencing data, Sanger sequencing or pyrosequencing on DNA extracted from different tissues were applied. None of the pathogenic variants was originally detected by Sanger sequencing on blood DNA.

Patient 1 displays an unusual combination of clinical features: he is cognitively only mildly affected, but shows severe limb reduction defects. The second patient presents with a moderate phenotype. Interestingly, Sanger sequencing analysis on fibroblast DNA did not detect the disease-causing variant previously observed on the same DNA sample by exome sequencing. Subsequent analyses could confirm the variants by Sanger sequencing on buccal mucosa DNA. Notably, this is the first report of a higher mutational load in buccal mucosa than in fibroblast cells of a CdLS patient.

Detection of low-level mosaicism is of utmost importance for an accurate molecular diagnosis and a proper genetic counseling of patients with a clinical diagnosis of CdLS. Next-generation sequencing technologies greatly facilitate the detection of low-level mosaicism, which might otherwise remain undetected by conventional sequencing approaches.

Keywords: Cornelia de Lange syndrome, *NIPBL*, mosaicism

Introduction

Cornelia de Lange syndrome (CdLS, OMIM #122470, 300590, 610759, 300882, and 614701) is a rare developmental disorder, clinically and genetically heterogeneous. Besides the distinctive facial features, the syndrome is characterized by growth retardation, cognitive delay with behavioral problems and multiple congenital abnormalities (Kline et al., 2007). To date, approximately 75% of patients with CdLS carry mutations in structural components (*SMC1A*, *SMC3*, *RAD21*) or regulators (*NIPBL*, *HDAC8*) of the cohesin complex (Krantz et al., 2004; Tonkin et al., 2004; Musio et al., 2006; Deardorff et al., 2007; Deardorff et al. 2012a,b). Cohesin plays a fundamental role in the maintenance of genomic stability, since it is involved in the regulation of sister chromatid cohesion, gene expression, DNA repair and chromatin remodeling (Watrin and Peters, 2009; Merkenschlager, 2010; Dorsett and Strom, 2012). In addition to cohesin, variants in other chromatin regulators such as *ANKRD11*, *AFF4*, *EP300*, *KMT2A*, *SETD5* or subunits of the SWI/SNF chromatin-remodeling complex were described in nearly 1% of patients with CdLS (Woods et al., 2014; Izumi et al., 2015; Yuan et al., 2015; Parenti et al., 2016a; Parenti et al., 2017). Notably, pathogenic variants in all these genes have been described as genetic causes of other developmental syndromes which share phenotypical features with CdLS, thus indicating that chromatin dysregulation is crucial for understanding the pathogenesis of this group of clinically overlapping disorders.

Despite the newly identified causative genes, *NIPBL* remains the major CdLS gene (Mannini et al.,2013), with disease-causing variants detected in nearly 65% of CdLS patients. Mutations in *NIPBL* tend to be associated with more severe phenotypes and with a higher frequency of limb reduction defects in comparison to variants in other cohesin-related genes (Deardorff et al., 2012a,b; Gervasini et al., 2013; Kaiser et al., 2014; Gil-Rodriguez et al., 2015; Parenti et al., 2016b). However, the portion of *NIPBL* mutations even increases by taking into account the role of somatic mosaicism. Mosaic variants in *NIPBL* were identified in more than 20% of

individuals in a cohort of patients originally reported to be mutation-negative by conventional Sanger sequencing approaches on DNA isolated from blood samples (Huisman et al., 2013). These mosaic variants are of particular interest, because they often escape routine molecular diagnostics, since the sensitivity of Sanger sequencing only allows detection of 15-20% of aberrant alleles or because of a focused molecular diagnostics restricted to blood DNA samples.

Here we will discuss and expand the crucial role of somatic mosaicism in CdLS and present two additional patients with mosaic disease-causing variants in *NIPBL*.

Somatic mosaicism

Somatic mosaicism is a well-known biological phenomenon, occurring not only in humans, but also in other species. It indicates existence of at least two different genotypes in the same individual and can occur for any type of mutation, either at the chromosomal or DNA sequence level. It is difficult to predict clinical consequences of mosaicism, as they depend on the timing of the mutation events, type of mutation, percentage of mutated cells and tissue distribution of the genetic change. Patients with mosaic mutations sometimes show milder phenotypical presentation of the disease, but are also often not distinguishable from those patients with non-mosaic heterozygous variants. In other cases, mosaic individuals appear healthy, without any distinguishable phenotype and mosaicism is revealed only upon molecular testing. Many alterations can occur only in a mosaic form, as the mutation is lethal when present in every cell (Spinner and Conlin, 2014). In favor of this hypothesis speaks the fact that only ~30% of conceptions result in live births and that majority of pre- and post-implantation pregnancy losses are associated with chromosomal abnormalities in the gametes and embryo (Macklon et al., 2002).

Mosaic mutations can be present in somatic, germinal cells or both, but typically only mutations in the soma have phenotypic consequences and can be detected by current

sequencing techniques. Germline mosaicism is instead speculated only in the presence of several affected offspring of apparently unaffected parents. However, molecular detection of germline mosaicism is limited by inability to obtain germline DNA, especially in females (Youssoufian and Pyeritz, 2002; Freed et al., 2014).

The first mosaic chromosomal abnormality was reported in 1960 (Hirschhorn et al., 1960), while the first suggestion of chromosomal mosaicism in patients with CdLS was reported in 1965 (Payne and Maeda, 1965). In this report, translocations and deletions were visible only in some of the analyzed karyotypes of patient cells, while the other cells appeared normal. The first patient with a mosaic mutation in *NIPBL* was reported by Castronovo and colleagues (Castronovo et al., 2010). Since then, somatic mosaicism has been described for all five CdLS genes, though the vast majority affects *NIPBL* (Hoppman-Chaney et al., 2012; Slavin et al., 2012; Huisman et al., 2013; Braunholz et al., 2015; Ansari et al., 2014; Gil-Rodriguez et al., 2015; Parenti et al., 2016b). The phenotypical spectrum of patients with mosaic variants in *NIPBL* is very broad, ranging from mildly to severely affected individuals. Therefore, distinct clinical features indicating somatic mosaicism have not been described so far.

Interestingly, very low levels of somatic mosaicism for some of the CdLS genes have been reported also in few unaffected parents of patients with CdLS: Slavin and colleagues described low-levels of a mosaic mutation in *SMC1A* in the blood DNA of an unaffected father of two affected sisters (Slavin et al., 2012). In addition, our group recently reported an unaffected mother of two siblings with CdLS, mosaic for an *HDAC8* variant in her urine (19%) and blood DNA (2%) (Parenti et al., 2016b). Few cases with gonadal *NIPBL* mosaicism have also been described (Niu et al., 2006; Weichert et al., 2011; Slavin et al., 2012). However, a recent study showed that a portion of *de novo* mutations presumed to be germline actually either occurred post-zygotically in the embryo or were inherited from the mother or father with low-level mosaicism (Acuna-Hidalgo et al., 2015).

Here we report two patients with the clinical diagnosis of CdLS, who were found to be negative for pathogenic variants in the five CdLS genes by conventional Sanger sequencing approaches on blood DNA. The disease-causing variants were subsequently identified by means of next generation sequencing techniques.

Patients

Patient 1 (Fig. 1a) is a 32 years old German male being the first child born to healthy parents. The pregnancy was uneventful and he was born after 42 weeks of gestation. Birth length was 48 cm (-2.25 SD), birth weight 3170 g (-1.35SD), occipital frontal circumference (OFC) 32 cm (-2.93 SD), and Apgar score were 6/9/10, respectively. He has not displayed any feeding difficulties. The patient presents with facial features typical for CdLS, namely synophrys, long eyelashes, depressed nasal bridge, upturned nose, thin upper lip, low set ears and low anterior hairline. He also presents with split hand deformity on the left hand and ulnar oligodactyly with missing fifth finger on the right hand. His left leg is 1.3 cm shorter than the right leg, due to pronounced hip dysplasia, corrected by orthopedic shoes. He was able to sit alone at the age of 9 months and to walk independently at the age of 20 months. He spoke his first words when he was almost one year old and he is able to communicate by using only a few words, but he is not able to produce longer sentences. The patient is capable to write and read with certain limitations, to swim, ride a bike, solve puzzles and to use modern media such as a digital camera. He is able to take care of himself and works in a carpenter´s shop. He occasionally suffers from headaches and has excessive amount of saliva.

At the current age (32 years), the patient's height is 156 cm (-0.8 SD), his weight is 40 kg (-0.3 SD) and his OFC is 52.8 cm (-2.4 SD).

Buccal DNA of the patient was analyzed by targeted CdLS-panel, which was performed on the Personal Genome Machine (Ion Torrent PGM, Life Technologies, Darmstadt, Germany)

as previously described (Braunholz et al., 2015). By this, we identified a nonsense mutation in *NIPBL* (RefSeq NM_133433), namely c.4399A>T; p.(Lys1467*), in 30% of the sequencing reads. The variant was confirmed by Sanger sequencing on buccal DNA, as well as on DNA obtained from epithelial urine cells, whereas it was not detectable on blood DNA (Fig. 1c). Parents were not available for testing, but the variant was not present in the Genome Aggregation Database (gnomAD).

Patient 2 (Fig. 1b) is a female, the only child born to a German mother and a Syrian father. Family history comprises Lupus erythematosus and vitamin D deficiency. Additionally, the mother had terminated a previous pregnancy due to the presence of trisomy 21. The pregnancy of the index patient was unremarkable, albeit prenatal brachycephaly was described. The delivery was vacuum-assisted, birth weight was 3140 g (-0.79 SD), birth length was 50 cm (-0.77 SD), occipital frontal circumference (OFC) was 35 cm (0.08 SD), Apgar scores were 9/9/10 and pH of umbilical arteries was 7.18. The patient showed early feeding difficulties, which required a high-caloric diet. She was able to walk independently at 18 months. She was diagnosed with patent foramen ovale with right to left shunt and X-ray of her hands showed shortening of the first metacarpal. She presents with a friendly behavior. Array-CGH, MLPA for *NIPBL* and Sanger sequencing on blood DNA for *NIPBL*, *SMC1A*, *RAD21* and *HDAC8* could not detect any disease-causing variant. For this reason, the patient was selected to undergo a trio-based exome sequencing analysis, which was performed on the HiSeq 2500 system (Illumina, San Diego, CA, USA) as previously described (Parenti et al., 2017). This analysis, which was performed on DNA extracted from fibroblasts, led to the identification of the *de novo* missense mutation c.5483G>A, p.(Arg1828Gln) in *NIPBL* in 15% of the sequencing reads. The variant was not present in the Genome Aggregation Database (gnomAD). Interestingly, we were not able to confirm this mutation by Sanger sequencing neither on blood nor on fibroblast DNA (Fig. 1d). However, the electropherogram performed on fibroblasts displayed a small mutant peak underneath the wild type base. As

next, we performed pyrosequencing on blood and fibroblast DNA of the patient. The ratio between the mutant and the wild type allele was found to be 14:86 on fibroblasts DNA and 2:98 on blood DNA (Fig. 1e), hence explaining why the mutation was not detected by Sanger sequencing. We subsequently obtained DNA from a third tissue, namely buccal mucosa. In this tissue, the pathogenic-variant could be detected by conventional Sanger sequencing (Fig. 1d), and pyrosequencing analysis revealed a mutant:wild type ratio of 17:83 (Fig. 1e). We previously demonstrated that fibroblasts represent the best source of DNA for mutation detection through Sanger sequencing (Braunholz et al., 2015). Thus, our patient represents the first case in which cells from buccal mucosa show a higher percentage of the mutant allele than fibroblasts.

Discussion

Somatic mosaicism has been proven to play a very important role in the pathogenesis of several disorders (Freed et al., 2014). However, patients with mosaic mutations show variable phenotypes and it is not possible to predict in advance, based on clinical features, if a mutation will be found in a mosaic state or not. Thus, there is usually no clear correlation between the mutation load and the phenotype. For instance, severe limb reduction defects as seen in patient 1 are restricted to CdLS patients with variants in *NIPBL* and almost exclusively caused by truncating variants or null alleles. While the severe malformation of the left arm is in agreement with the genotype, a mild cognitive impairment as seen in patient 1 is rather unique and has never been reported in a patient with this type of *NIPBL* mutation. Based on our findings that indicate absence of the mutation in blood and only 30% in buccal mucosa DNA, it is tempting to speculate that some other tissues, especially neuronal tissues, might show only small portions of the variant, which may explain the mild cognitive impairment. In a previous report another mildly affected CdLS patient with severe limb defects was reported as negative for a disease-causing variant in *NIPBL* using DNA from

lymphocytes [Bhuiyan et al., 2006]. Based on our findings it is tempting to speculate if this patient is also mosaic for a disease-causing variant in *NIPBL*.

Blood leukocytes have been the gold standard material for molecular analysis for decades. Nevertheless, mosaic mutations are frequently not detected in these samples, meaning that DNA of additional tissues is required for a comprehensive and proper molecular testing, especially of those patients with clear clinical diagnosis, but negative results. Previously, we have described three patients with somatic variants in *NIPBL* identified by conventional Sanger sequencing approaches on DNA extracted from fibroblast cells, but not on DNA from buccal mucosa or blood cells (Braunholz et al., 2015). Therefore, fibroblasts from skin biopsies were recommended as the most suitable tissue for molecular diagnostics. In contrast to our previous report, here we describe a somatic mosaicism that was detected by conventional Sanger sequencing on DNA from buccal mucosa, but not detected using fibroblast DNA. However, highly sensitive pyrosequencing approaches could identify the pathogenic variant in DNA samples of both tissues. Based on these findings, we recommend the use of sensitive next generation sequencing approaches with high numbers of sequencing reads that should not be restricted to DNA from blood samples. Only the combination of sensitive sequencing technologies and DNA from sources other than blood allow an appropriate molecular diagnostics of patients with clinical diagnosis of CdLS.

The identification of low-level mosaicism is of great importance for an accurate diagnosis and proper genetic counseling. Therefore, we strongly recommend integration of modern and highly sensitive sequencing techniques into the routine molecular diagnostics of CdLS. With increasing costs and availability of next generation sequencing technologies, we believe that high-coverage exome sequencing will be the most efficient and most suitable technology for appropriate molecular diagnostics of CdLS. Furthermore, to confirm and quantify putative mosaic variants represented by small numbers of total sequencing reads, we suggest additional sensitive targeted sequencing technologies such as pyrosequencing.

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Figure titles and legends: Figure 1. Phenotype of the patients and sequencing results. (a) Phenotype of patient 1, with a detail of the anomalies of the upper limbs. (b) Facial features of patient 2, including a detail of the small and widely spaced teeth. To be noted the presence of hirsutism.(c-d) Sanger sequencing of DNA obtained from different tissues ofpatient 1 (c) and patient 2 (d). (e) Pyrosequencing results from different tissues of patient 2.

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