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Split hand-foot malformation, tetralogy of Fallot, mental retardation and a 1 Mb 19p deletion-evidence for further heterogeneity?

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Abstract

Congenital limb malformations are the second most common birth defects observed in infants. Split hand foot malformation (SHFM), also known as central ray deficiency, ectrodactyly and cleft hand/foot, occurs isolated or in combination with other malformations.

We report a male patient with SHFM, tetralogy of Fallot and a clinical phenotype of Angelman syndrome. Using array based genome analysis (3K BACs and 500K SNPs), we identified a *de novo* deletion of chromosome 19p13.11, confirmed by Fluorescent *In Situ* Hybridization analysis. The deletion is 0.99 Mb in size and contains 28 genes. The proximal breakpoint of the deletion is in *EPS15L1*, which may be involved in vertebrate limb development.

Subsequent screening of 21 syndromic and non-syndromic SHFM patients (*TP73L* mutation negative) for rearrangements using Multiplex Ligation-dependent Probe Amplification did not detect other deletions or duplications in chromosome 19. These findings suggest that our patient may have a new contiguous gene syndrome and indicates that SHFM is genetically more heterogeneous than currently known.

Key words

Mental retardation - Split Hand Foot Malformation - Tetralogy of Fallot- -19p deletion

Introduction

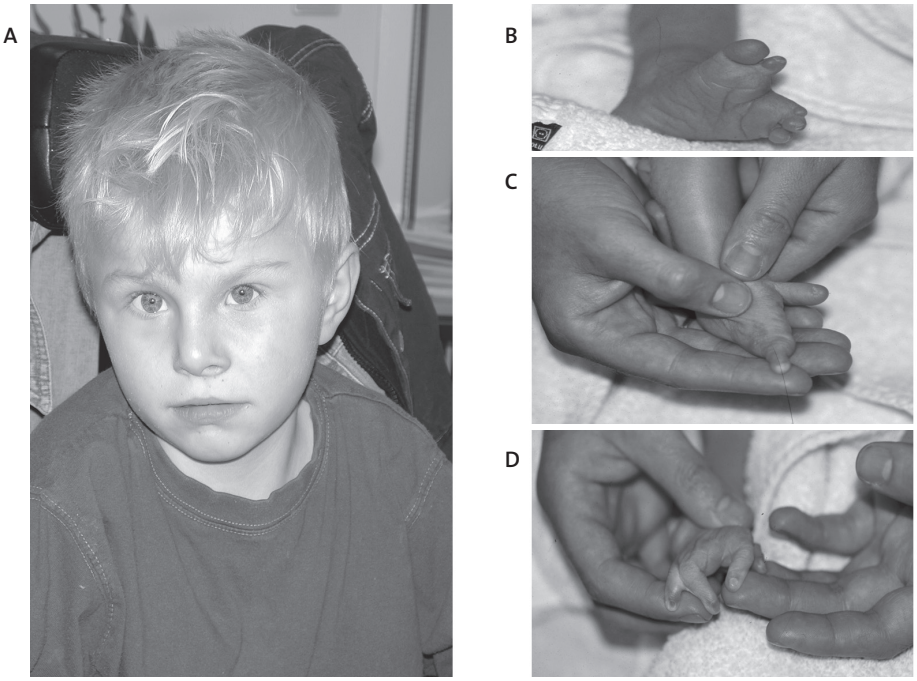
The variability of Split Hand Foot Malformation (SHFM) causes the classification to be very difficult. The hand and foot abnormalities range greatly in their phenotypic spectrum from mild abnormalities (i.e. digital shortness) to more severe abnormalities (i.e. adactyly), occurring in a single or in all four limbs. SHFM occurs either as an isolated malformation ('non-syndromic') or in association with other malformations ('syndromic'). To date several syndromic forms have clinically been identified (EEC OMIM#129900, EEM OMIM#225280, LMS OMIM#603543, MMEP OMIM#601349, KNS OMI M#183800 and ADULT OMIM #103285). Identification of genes involved in defects in human limb patterning has not yet been very successful, in part, by the enormous spectrum of phenotypes.

For the development of the limb, specialized cell clusters are important. The SHFM phenotype is believed to be caused by an underlying defect of one of the cell clusters named the apical ectodermal ridge (AER). Both genetic defects and environmental factors may cause SHFM by interfering with the AER [Duijf et al., 2003; Seto et al., 1997]. Because of expression in the AER, candidate genes in the different loci have been postulated but mutations have not yet been identified. Currently five genomic loci, based on case and family studies, have been implicated. They are known as SHFM1 (chromosome region 7q21-q22), SHFM2 (Xq26), SHFM3 (10q24), SHFM4 (3q27), and SHFM5 (2q31). Molecular testing is only available for SFHM4 since most EEC syndrome patients and 5-10% of non-syndromic SHFM patients have been shown to carry mutations in the *TP73L* (*p63*) gene [Duijf et al., 2003; de Mollerat et al., 2003; Brunner et al., 2002].

So far copy number variation (CNV, i.e. the presence of deletions or duplications) in patients with SHFM has only been described in SHFM1, SHFM3 and SHFM5. For SHFM1 only deletions have been described, mostly resulting from complex rearrangements and unbalanced translocations [Scherer et al., 1994; Bernardini et al., 2008; Sharland et al., 1991]. To explain inconsistent translocation/inversion breakpoints and failure to identify mutations in the candidate genes, a position effect has been postulated. For SHFM3 only duplications of 10q24 have been described. Extensive study of the candidate gene Dactylin did not reveal how the duplication leads to the SHFM3 phenotype [Everman et al., 2006; de Mollerat et al., 2003]. SHFM5 has been associated with interstitial deletions suggesting removal of a 'digit enhancer' or genes involved in the AER [Boles et al., 1995; Goodman et al., 2002; Bijlsma et al., 2005]. For SHFM, no large deletion or duplication events on other chromosomes have been described.

Here, we report a patient who manifested SHFM and who was found to have a 0.99 Mb deletion on chromosome 19.

Figure 1: Clinical features of the index patient. (A) Phenotype of the patient (here six years old) with subtle facial features including a wide mouth, fair hair and light skin. Central ray deficiencies as detected at birth. Left foot (B) with a cleft, hypoplasia of the second digit and a nubbin representing the third digit. Right foot (C) with a proximally placed first digit and presence of digit V. Digits II-IV are absent. Right hand (D) with a central cleft leading to absence of the third digit. Digit IV and V show cutaneous syndactyly.



Clinical report

The male patient (Fig. 1A) was born at term after an uneventful pregnancy. He is the second child of healthy unrelated parents. Family history for SHFM or mental retardation was negative.

At birth, congenital malformations of all extremities were conspicuous. Examination revealed a central ray deficiency of both hands and ectrodactyly of both feet. (Fig. 1B and 1C) The left-hand showed preaxial polydactyly with a bifid thumbnail, absence of the third digital ray and cutaneous syndactyly of the first and second digit and the fourth and fifth digit. The right-hand was a split hand with central ray deficiency and a cutaneous

syndactyly of the fourth and fifth digit (Fig.1D). The left foot showed a split foot with a hypoplastic second digital ray and a rudimentary third digital ray. There was cutaneous syndactyly of the third and fourth digital ray. The right foot showed ectrodactyly where only the first and fifth digital rays where present. The first digital ray was positioned far too proximally. A Ventricular Septum Defect (VSD) and stenosis of the pulmonal artery was suspected, which was later diagnosed as a tetralogy of Fallot. At the time of re-evaluation in the clinical genetics department the patient was two years old. His length was 88 cm (-0.3 SD) and head circumference 47 cm (-1 SD). Physical examination showed few dysmorphic features, brachycephaly with a flat occiput, slight upslant of his eyes, and broad mouth with a thin upper lip. He had very fair hair. His psychomotor development was slightly behind but this was considered to be normal given his physical problems early in life. Surgical correction of both the SHFM and the tetralogy of Fallot had taken place. Corrective surgery for strabismus was planned. MRI of the cranium and ultrasound of the kidneys was performed but showed no aberrations. EEG did not show epileptiform activity. The patient was re-evaluated at the age of six. By this time he had a severe delay in psychomotor development. His speech was severely impaired (4-5 words). He showed characteristic behavior with frequent laughing. Together with the previously described dysmorphic features this strongly suggested to the diagnosis of Angelman syndrome. Karyotyping did not reveal any chromosomal abnormalities (46, XY). M-FISH was normal and mutation screening for the *TP73L* gene as well as the *UBE3A* gene (causing Angelman syndrome) were negative. Also, deletions or methylation defects of *UBE3A* or UPD of chromosome 15 could not be detected. Evaluation of the patient at the current age of 10 years did not show any significant changes in phenotype.

Materials and methods

Subjects

The index patient was referred to our department for genetic counseling. His phenotype was established by examination of a clinical geneticist. Informed consent for genetic analyses was obtained from both his parents. Molecular testing was performed on whole blood genomic DNA.

Mutation analysis of the *UBE3A* gene and the *TP73L* gene were performed in diagnostic laboratories in the Netherlands (Erasmus MC Rotterdam, Radboud MC Nijmegen).

21 syndromic and non-syndromic SHFM patients from the Department of Clinical Genetics (Radboud MC Nijmegen) were ascertained for a MLPA based study and collected on the basis of clinical (and radiographic) findings. Patients were known to be negative for

TP73L mutations and had normal karyotypes. Array based genome analysis has not been performed on these patients. Informed consent was obtained from all participants.

DNA analysis

DNA was isolated from blood samples using standard methods. DNA concentrations were measured using a nanodrop.

Fish

Fluorescence in situ hybridization (FISH) was performed following standard protocols [Dauwerse et al., 1992]. The PAC/BAC clones RP11-413M18, CTD-2231E14 and CTD-3149D2, located at 19p13.11, were used for confirmation of the deletion. Clones were obtained from the Welcome Trust Sanger Institute, Cambridge, UK.

Array platforms

For array-comparative genomic hybridization (CGH), 1Mb spaced large insert clone arrays were used [Knijnenburg et al., 2005]. This platform contains ~3500 large insert clones spotted in triplicate at approximately 1Mb density over the full genome. The clone set was distributed by the Wellcome Trust Sanger Institute. All laboratory processing and hybridizations were performed according to published protocols [Fiegler et al., 2003].

The Affymetrix 500K oligonucleotide array was used according to manufacturer's protocols (http://www.affymetrix.com). Regions of copy number gain and loss were detected using the hidden markov model output of CNAG 2.0 [Nannya et al., 2005]. Deletions detected were described according to the recommendations of the HGVS (http://www.hgvs.org/mutnomen/).

Multiplex Ligation-dependent Probe Amplification

In total, 22 MLPA probe pairs were designed for genes of interest in and outside the deleted 19p region (Table 1).The probes were divided between two probe sets. Within each probe set, control probes for unlinked loci were included as a reference. Sequences are available on request. Peak ratios between 0.75 and 1.25 are considered normal (i.e. two copies).

Probe design and the MLPA reaction and analysis were performed as described [White et al., 2004; Schouten et al., 2002] Genescan (Applied Biosystems) and Genemarker 1.51 (Softgenetics) were used for data analysis.

Table 1: Genes on Chromosome 19p13.11

RefSeq DNA ID	Gene Name	Mlpa probe	Gene Start (bp)	Gene End (bp)
NM_032493	AP1M1	X	16169731	16207149
NM_016270	KLF2	X	16296637	16299683
NM_021235	EPS15L1	X	16333408	16443762
NM_145046	CALR3	X	16450888	16468003
NM_032207	C19orf44	X	16468205	16493163
NM_006387	CHERP	X	16489700	16514263
NM_024881	SLC35E1	X	16523584	16544193
NM_004831	MED26	X	16546718	16600015
NM_024104	C19orf42	X	16617967	16631968
NM_024074	TMEM38A	X	16632938	16660814
NM_001007525	NWD1		16703001	16789756
NM_015260	SIN3B	X	16801218	16852164
NM_003950	F2RL3	X	16860826	16863830
NM_015692	CPAMD8	X	16864765	16998625
NM_033417	AC020908.7		17021573	17047343
NM_004145	MYO9B	X	17073466	17185093
NM_018467	USE1		17187155	17191638
NM_024578	OCEL1	X	17198055	17201027
NM_005234	NR2F6	X	17203694	17217151
NM_031941	USHBP1	X	17221838	17236573
NM_014173	C19orf62		17239232	17251162
NM_152363	ANKLE1	X	17253454	17259455
NM_024527	ABHD8	X	17263941	17275282
NM_023937	MRPL34	X	17277477	17278652
NM_024050	DDA1		17281350	17292098
NM_020959	TMEM16H		17295034	17306638
NM_133644	GTPBP3		17309379	17314530
NM_031310	PLVAP		17323264	17349159
NM_004335	BST2	X	17374750	17377401
NM_138401	FAM125A		17391856	17397140
NM_138454	NXNL1		17427236	17432725
NM_198580	SLC27A1	X	17442300	17477977

Genes inside the deleted 19p11 region (indicated in yellow) and outside the deleted region (indicated in white). MLPA probes were designed for 22 genes (human reference sequence NCBI Build 36.1)

Results

Index Patient

Array-CGH revealed a deletion of 1 BAC clone RP11-413M18. Flanking clones (CTD-3149D2, CTD-2231E14) on chromosome19p13.11 were not deleted, giving an estimated maximal deleted size of 1.44 Mb (Fig. 2A).

FISH analysis using RP11-14M18 confirmed the deletion in chr.19p13.11, with flanking probes CTD-3149D2 centromeric and CTD-2231E14 telomeric giving normal signals (data not shown).

To determine the extent of the deletion more precisely, a 500 K oligonucleotide array (Affymetrix) was used. This array showed a deletion (rs12460131_rs10411936)_(rs4808641_rs6512194). Rs10411936, the first deleted SNP, is positioned in an intron of *EPS15L1*. Rs4808641, the last deleted SNP, is a SNP localized between two genes (*FAM125A* and a novel gene). The deletion spans ~ 0.99 Mb and contains 28 genes (Fig. 2B).

Figure 2: (A) Array-CGH showed one deleted BAC clone (RP11-413M18) on 19p13.11 at 16.8 Mb with a maximal deleted size of 1.44 Mb. **(B)** SNP array refined the deletion from rs10411936 (19: 16,409,375) - rs4808641 (19:17,408,292) with a maximal deleted size of 0.99 Mb

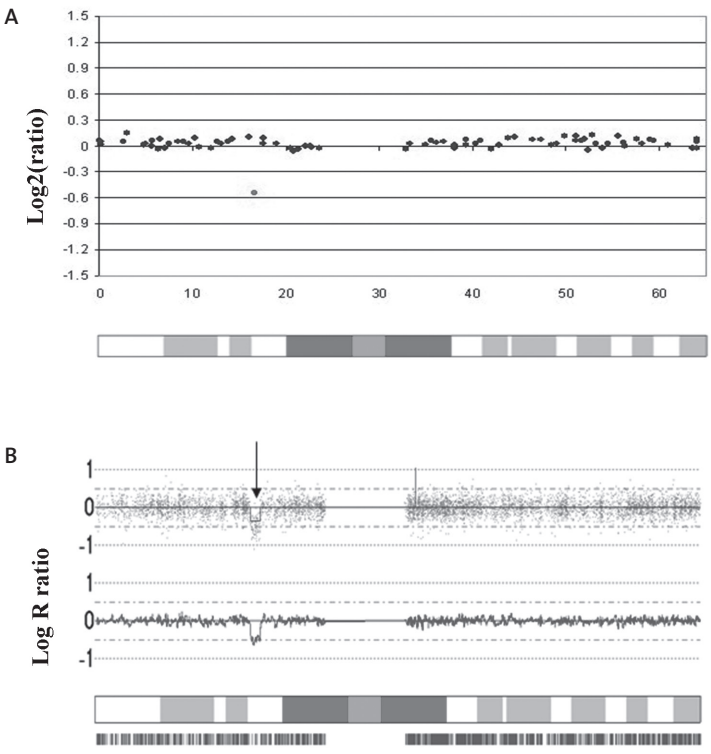
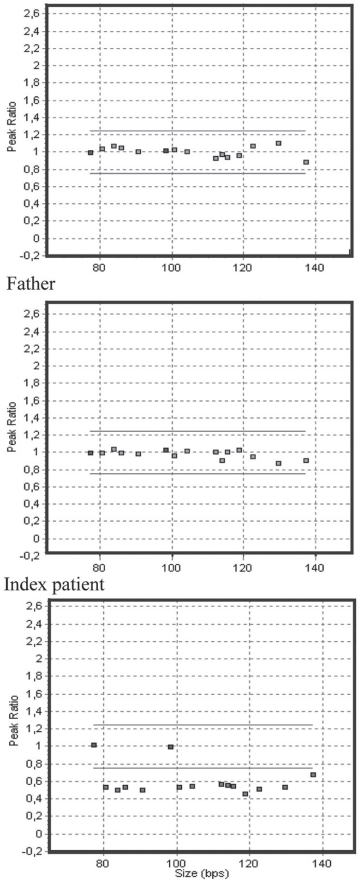
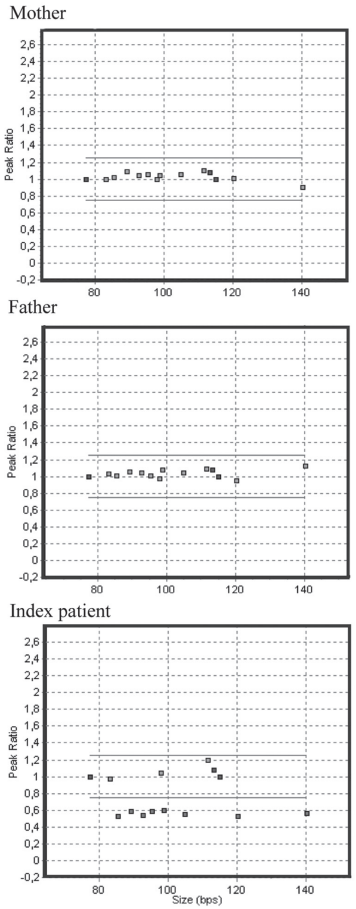


Figure 3: MLPA results for the index patient and his parents. **(A)** Peak ratio plots for the two probe sets, confirming the deletion to be *De Novo*. Control probes are represented by blue squares. **(B)** Peak ratio results for the mother (1), the father (2) and the index patient (3). Peak ratios in the index patient are normal for probes located in KLF2, AP1M1 and SLC27A1, coinciding with the proximal and distal deletion breakpoints.

A



B



		REF -	1	2	3
1	*control1	77.2	0.996	0.989	1.016
2	ANKLE1_1	80.4	1.014	0.970	0.520
3	ANKLE1_2	83.8	1.066	1.037	0.501
4	CPAMD8	86.0	1.044	0.990	0.526
5	EPS15L1	90.6	1.000	0.979	0.500
6	*control2	98.5	1.018	1.026	0.995
7	MYO9B	100.7	1.030	0.962	0.534
8	MED26	104.2	1.000	1.018	0.541
9	USHBP1	112.1	0.923	0.999	0.570
10	ABHD8	114.1	0.969	0.904	0.559
11	CALR3	115.6	0.917	0.979	0.538
12	BST2	118.9	0.952	1.012	0.456
13	TMEM38A	122.6	1.071	0.943	0.509
14	C19ORF44	129.8	1.088	0.866	0.529
15	SLC35E1	137.6	0.881	0.898	0.671

		REF -	1	2	3
1	*control1	77.4	1.000	1.000	1.000
2	KLF2	83.4	1.016	1.051	0.991
3	F2RL3	85.5	1.097	1.083	0.566
4	ANKLE1	89.5	1.102	1.069	0.590
5	SIN3B	92.9	1.088	1.089	0.567
6	CHERP	95.3	1.086	1.041	0.605
7	SLC27A1	97.9	1.016	0.988	1.053
8	OCEL1	99.0	1.053	1.089	0.601
9	NR2F6	105.0	1.070	1.059	0.563
10	AP1M1	111.6	1.106	1.094	1.196
11	*control2	113.2	1.076	1.072	1.072
12	*control3	115.2	1.000	1.000	1.000
13	MRPL34	120.3	1.024	0.970	0.534
14	C19ORF42	140.3	0.898	1.113	0.560

To study the deletion on chromosome 19p13.11 in more detail and to facilitate the screening of a larger set of patient samples, we designed a MLPA kit for twenty-two genes in the 19p13.11 region. MLPA analysis confirmed the deletion on chr. 19p13.11 in our patient. The deletion was not present in either parent, so proven to be *de novo* (Fig. 3A). Genes located proximal (*KLF2, AP1M1*) and distal (*SLC27A1*) from the deleted region were present in two copies in the index patient (Fig 3B).

SHFM patients

Twenty-one SHFM (TP73L mutation negative) patients were collected to asses whether copy number variation on chromosome 19p13.11 occurred more frequent in this patient group. The MLPA kit was used for additional screening of rearrangements in the 19p13.11 region. This did not reveal copy number changes in any of the tested patients.

Discussion

We present a case study in which a *de novo* deletion of chromosome 19p13.11 was found. The patient we report has, apart from SHFM, a congenital heart defect and mental retardation with a clinical phenotype suspected of Angelman syndrome. None of the genes within this deletion could obviously be related to the phenotype. Since the SHFM was such a salient feature in our patient, we decided to focus on limb malformations. This is the first case where a deletion on chromosome 19 has been associated with SHFM. The proximal breakpoint of the *de novo* deletion is in *EPS15L1*, a gene coding for epidermal growth factor receptor pathway substrate 15-like 1. The encoded protein seems to be a constitutive component of clathrin-coated pits that is required for receptor-mediated endocytosis. EPS15L1, like its homologue Eps15 on chromosome 1, functions as a substrate for the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) [Wong et al, 1995].The epidermal growth factor receptor signaling pathway is one of the most important pathways that regulate growth, survival, proliferation and differentiation. It has been implicated in vertebrate and invertebrate limb development, including the AER [Simcox et al., 1997; Omi et al., 2005; Clifford et al., 1989]. Moreover, there is evidence that p63 regulates EGFR expression [Nishi et al., 2001], suggesting *EPS15L1* to be a serious candidate gene for SHFM. However, no copy number changes in *EPS15L1* were found in any of the other SHFM patients suggesting that a deletion or duplication of this gene is not frequently involved in the pathogenicity of SHFM. Nevertheless, point mutations as a possible mechanism can not be excluded.

To exclude Copy Number Variations in the SHFM1-SHFM5 loci as a possible cause for the ectrodactyly, both our index patient and the 21 SHFM patients were tested with MLPA. A candidate gene in each SHFM locus (*DSS1, FGF13, FBXW4, TP73L, DLX2*) was tested. Our patient did not show any dosage abnormalities in these genes.

A previous linkage study on EEC syndrome in a large Dutch family originally mapped a gene to a region of chromosome 19 comprising 19p13.11 [O'Quinn et al., 1998]. However, a causative mutation in the *p63* gene on chromosome 3q27 was later identified in this family, which refuted the presence of an SHFM locus on chromosome 19 [van Bokhoven et al., 2001]. However, the possibility of a chromosome 19 linked modifier effect in this family cannot be excluded. Copy number changes in chromosome 19p13 have been described by Thienpont et al., et al. using array-CGH to study patients with congenital heart defects [Thienpont et al., 2007]. They describe a patient (DECIPHER CHG00001031) with a *de novo* duplication (19) (p13.12-p13.11) which has recently been shown to be a more complex intrachromosomal rearrangement including a microduplication and two microtriplications spanning 3.2 Mb [Thienpont et al., 2008]. A small part of the first triplication and the major part of the duplication overlaps the region deleted in our patient, thus containing the same genes. Interestingly, the clinical phenotype of mental retardation, microcephaly, muscular VSD, short stature, strabismus and speech delay partly resembles the phenotype of our patient. However, the lack of overlap in facial dysmorphism and the absence of SHFM are under dispute. In general in microdeletion/duplication syndromes, phenotypes corresponding to the duplication are different and milder than those found in deletion cases. In OMIM none of the 19p13.11 deleted genes in our patient have been implicated with disease. Only a common variant located in the *MYO9B* gene has been associated with celiac disease [Monsuur et al., 2005].

At present, the interpretation of detected copy number changes is not straightforward, given the fact that as much as 11% of our genome shows copy number variations that do not appear to be clearly disease-related [Redon et al., 2006]. To distinguish pathogenic from innocent variants not only data from patients should be collected, but especially those found in 'normal' individuals. The Database of Genomic Variants (http://projects.tcag.ca; version April 2008), in which variants not known to cause disease are collected, reports one case with a duplication on chromosome 19 (19:16,998,070-16,999,171) that lies within our deleted region [de Smith et al., 2007]. The duplicated gene *CPAMD8* encodes the C3 and PZP-like, alpha-2-macroglobulin domain containing 8. This protein belongs to a family of the complement component-3, involved in innate immunity and damage control. No other genes within our deletion have been reported in the Database of Genomic Variants.

Elucidating the underlying cause of known and new syndromes remains highly challenging.

Copy Number Variants can impact gene function in several ways. The formation of a fusion gene as a possible pathogenic mechanism is not plausible because the distal border of our deletion is intergenic. The phenotype of our patient may be due to a dosage effect on one or multiple genes but it is still very likely that other mechanisms underlie pathogenicity such as disruption of a regulatory element that influences flanking genes. Notably, no miRNA's are encoded by genes in the deleted area.

Based on the phenotype of our patient, the size and high gene content of the deletion, the fact that it is *de novo* and its absence in the Database of Genomic Variants, we believe that this deletion is causative and represents a new contiguous gene syndrome. Moreover, our finding suggests SHFM to be even more genetically heterogeneous than previously suggested. Collecting larger sets of patient data in databases such as DECIPHER and ECARUCA may confirm this assumption, and should ultimately help to narrow down the critical region, and eventually pinpoint relevant genes or regulatory sequences.

Acknowledgements

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