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Distal limb deficiencies, micrognathia syndrome (OMIM 246560) and syndromic forms of split hand foot malformation (SHFM) are caused by chromosome 10q genomic rearrangements

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Running title: 10q24 microduplication in syndromic SHFM3

ABSTRACT

Background: The 10q24 chromosomal region has previously been implicated in Split Hand Foot Malformation (SHFM). SHFM3 was mapped to a large interval on chromosome 10q. The corresponding Dactylaplasia mouse model was linked to the syntenic locus on chromosome 19. It was shown that the two existing *Dac* alleles result from MusD-insertions upstream of or within *Dactylin (Fbxw4)*. However, all efforts to find the underlying cause for the human SHFM3 have failed on the anaysis of all the genes within the linkage region. Intriguingly a submicroscopic duplication within the critical locus on chromosome 10q24 was associated with the phenotype.

Methods and Results: As a part of screening for genomic rearrangements in cases with unexplained syndromic limb defects, a cohort of patients was analyzed by array CGH (Comperative Genomic Hybridization). A 10q24 microduplication was detected in 6 individuals with distal limb deficiencies associated with micrognathia, hearing problems and renal hypoplasia. In addition, in a family with two affected siblings, a somatic/gonadal mosaicism for the microduplication was detected in the apparently healthy mother. Using a high resolution oligoarray further delineation of the duplication size was performed.

Conclusions: The detected 10q24 genomic imbalance in our syndromic patients has a similar size to the duplication in the previously reported individuals with an isolated form of SHFM, thus extending the clinical spectrum of SHFM3. These findings clearly demonstrate the importance of array CGH in the detection of the etiology of complex, clinically heterogeneous entities.

INTRODUCTION

Split Hand Foot Malformation (SHFM) is a relatively rare anomaly characterized by central ray deficiencies and fusions of the remaining bones to variable degrees. Occasionally only remnants of the autopod are present (oligo/monodactylous ectrodactyly or peromelia). This condition represents 8-17% of all limb reduction defects and occurs in 1 in 20000 newborns [9, 12]. Non-syndromic and syndromic forms have been described and both can be familial or sporadic representing all possible types of inheritance. Reduced penetrance is often observed in affected families.

Until now, five SHFM loci have been mapped in humans: SHFM1 at 7q21.2q22.1; SHFM2 at Xq26; SHFM3 at 10q24q25; SHFM4 at 3q27; and SHFM5 at 2q31. Only for one of them (SHFM4) the causal gene, *p63* has been identified [3].

Using classical linkage analysis, the SHFM3 locus was originally mapped to 10q24, a region syntenic to mouse chromosome 19 [25, 27]. The fact that the mouse *Dactylaplasia* phenotype, caused by insertional mutations of *Fbxw4*, resembles SHFM in man made the genes within the linkage region good candidates for the human phenotype [29]. No causal sequence alterations have been found, although two interesting genes (*FGF8* and *FBXW4*) reside within the critical locus. Intriguingly a tandem duplication of 325-570Kb was detected in 17 familial and sporadic cases. All affected individuals had an isolated form of typical SHFM, except two. In one patient the phenotype was associated with medulloblastoma and another had a submucous cleft palate. Two genes, *BTRC* and *POLL*, were constantly included in the detected aberrations, whereas *FBXW4* was partially engaged [3, 9, 13, 16, 24].

In this study, a group of individuals with a variety of syndromic forms of reduction limb defects were selected for genomic screening with a home made 1Mb array CGH. A 10q24 genomic rearrangement was revealed in two siblings (C2755 and C224256), published elsewhere as having distal limb deficiencies-oral involvement-renal defect syndrome or classified in MIM as Distal limb deficiencies with micrognathia syndrome (MIM 246560) [7, 10]. Based on the clinical phenotype three additional cases were selected and they all did have similar genomic aberrations. Using a high resolution whole genome oligoarray, a fine mapping of the breakpoints was performed. The observed rearrangement was similar in size to those previously detected in non-syndromic SHFM3 patients but extended more distally in the *FGF8* direction. Two of the cases were familial- each with two affected sibs and healthy parents, the other two were sporadic. Only in one familial case the parents were available for analysis and a somatic mosaicism for this chromosome 10q24 duplication was detected in the mother.

PATIENTS AND METHODS

Patient's phenotypes

All patients were examined by experienced clinical geneticists and have normal high resolution G-banded karyotypes. Patient's phenotypes are summarized on Table 1. Extended clinical and family data are available in the Supplementary materials. For two patients, C2755 and C224256, mutations in the coding part of *OSR1* and *OSR2* genes have been excluded by direct sequencing [10]. Written consent form was obtained from all patients and/or their legal guardians for publication of images and clinical data in print and online.

In summary, the two affected siblings (C2755 and C224256) in Family 1 (F1) have severe truncation defects of all four limbs and facial dysmorphism. The more severely affected older brother (C2755) is mentally retarded. In addition he has severe myopia,

renal defects and hearing loss (Fig.1a-c). The parents were reported healthy but were not available for examination [7].

C211000 (F2) is the second child of healthy, unrelated parents. He has a phenotype similar to those of the siblings in F1 [19].

C365650 (F3) is a patient with classical four limb SHFM and renal hypoplasia (Fig.1k-1). His parents are healthy and unrelated.

C369374 and C369373 are the second and third child of healthy, unrelated parents (F4). The younger sister (C369373) has classical SHFM. The skeletal defects, facial gestalt (Fig.1d-j) and detected hearing loss of the more severely affected brother (C369374) resemble those of the patients published as Distal limb deficiencies with micrognathia syndrome (DLDMS) [7, 19].

| Patients Phenotype | C2755 | C224256 | C211000 | C369373 | C369374 | C365650 |
|---------------------------------|--|---|--|--|---|---------------------|
| Face | maxillary hypoplasia, micrognathia, high arched palate | maxillary hypoplasia, micrognathia, high arched palate | maxillary hypoplasia, micrognathia, cleft palate | axillary normal ypoplasia, iicrognathia, eft palate | | normal |
| Eyes | Severe myopia | normal | nystagmus, severe myopia | normal | normal | normal |
| Ears | low set ears, recurrent otitis in childhood, bilateral conductive hearing loss | low set ears | low set ears, recurrent otitis, bilateral conductive hearing loss, | normal | low set ears, recurrent otitis, bilateral mixed conductive- sensorineural hearing loss | normal |
| Upper limbs | Absent radii, hypoplastic curved ulnae, rudimentary carpal bones, absence of other bones of the hands | deformation and bowing of distal radii, absent carpal bones, missing 1 st right and 2 nd right and 1 st left metacarpals, one rudimentary dysmorphic bone between 3 rd and 4 th metacarpals, only one 5 th finger with three phalanges | normal long bones, two metacarpals, one postaxial (5 th) finger with two phalanges | classical SHFM | normal long bones, four metacarpals, one postaxial (5 th) finger with three phalanges, one phalanx crossing between 4 th and 5 th metacarpals and one rudimentary bone between 3 rd and 4 th metacarpals | classical SHFM |
| Lower limbs | normal long bones, bilateral synostosis of rudimentary calcaneus and talus, absent tarsal bones, one short and dysplastic tubular bone of both feet | normal long bones, talus, calcaneus, naviculare and cuboideum, rudimentary 1 st metatarsal, missing 2-4 metatarsals, 5 th metatarsal with large base and one two- phalangeal digit | normal long bones, one metatarsal, one postaxial digit with one phalanx | classical SHFM | classical SHFM | classical SHFM |
| Internal organ abnormalities | oligomega- nephronia | none | renal hypoplasia | none | none | renal hypoplasia |
| Mental development | moderate | borderline | normal | normal | normal | normal |
| Other abnormalities | undescended left testis | none | none | none | none | none |

Table1. Patient's phenotypes.

Legend: MR- mental retardation; SHFM- Split Hand Foot Malformation.

Patient's material

Peripheral blood cell DNA from patients and parents (Table. 2) was obtained after informed consent, following standard protocols.

Array CGH analysis

The screening was performed using a home made 1Mb array CGH platform. All hybridizations and the analysis were done as described [32].

For fine mapping of the breakpoints, the 244K oligoarray (Agilent) was used. All hybridizations and the analysis were performed according to the manufacturer's protocol, using a circular binary segmentation (CBS) algorithm for aberration detection and a smoothing window of 10 points [31]. Thus four abnormal consecutive clones were used to call a duplication, allowing 3.5% false negative results [Supplementary materials].

FISH analysis

FISH analysis was performed using probes within or flanking the detected 10q24.31q24.32 microduplication (Fig.4). Those were BAC RP11-108L07 and BAC RP11-774C22 labelled in SpectrumOrangeTM- dUTP (red signal); and BAC RP11-619N16, RP11-529I10 and BAC RP11-489E24 labeled in SpectrumGreenTM- dUTP (green signal), respectively. Micro-duplication/triplication testing of patients C2755 and C224256 was done on interphase nuclei. Fifty cells of each individual were scored. Direct FISH screening of one hundred peripheral white blood cells of individual C369371 was done for detection/confirmation of somatic mosaicism. The probe's labeling and hybridizations with small modifications were performed as described elsewhere [1].

Q-PCR analysis

SYBR Green qPCR assays have been designed for ABI 7500 sequence Detection System. All primers have been created using Primer Express software (Applied Biosystems, Foster City, CA). The genomic positions of each primer pair are shown on Supplementary Table 1. The uniqueness of the amplicons has been checked by UCSC *in-silico* PCR based on the March 2006 human reference sequence, NCBI Build 36.1 [18]. Efficiency of each assay was tested using serial dilutions of control DNA. All reactions were carried out with 10ng genomic DNA, in duplicate. Ct values were obtained by SDS2.0 system (Applied Biosystems) and comparison of relative quantities, calculated by $\Delta\Delta$ Ct method [23], was done. Single assay of *p53* gene was used for normalization. Each assay was repeated two times. Means of these two independent experiments, with standard deviation (SD) less than 0.15, were used for final fold difference calculation. All analyses were carried out in Excel (Microsoft Corp., Redmond, WA).

RESULTS

1Mb BAC array CGH screening

1Mb array CGH screening in a group of patients with unexplained syndromic forms of congenital skeletal anomalies revealed in two of them (C2755 and C224256) a gain of signal of three consecutive clones- RP11-324L3, RP11-529I10, RP11-264H19 (Supplementary Fig.1a and b). All three BACs were located on chromosome 10q24.31q24.32. The probands were originally published as having a new autosomal recessive syndrome [7]. Based on their phenotype- terminal limb reduction defects/SHFM in combination with facial dysmorphism and/or hearing problems,

and/or renal insufficiency- three additional cases were selected for array CGH analysis. All patients carried an aberration of the same region.

In 4/5 analyzed individuals the Log2 ratio of the abnormal clones corresponded to a duplication. However, one patient (C2755), in contrast to his less severely affected sister, had a Log2 ratio consistent with a triplication (Supplemental Fig.1a).

244K Agilent array

Since apparently simple genomic rearrangements were recently often found to be more complex [30], the extent of the duplication and the presence of additional cisand/or trans-copy number variations in this group of patients were further investigated. Therefore 10 individuals, including all affected members of the four families and the parents of one sporadic and one familial case, were analyzed with a 244K array CGH platform (Agilent).

No other common cis- or trans-copy number variations that can explain the more extended clinical features of our patients, have been detected. All affected individuals, except case C365650, carried a duplication of at least 500Kb, including five genes-*LBX1*, *BTRC*, *POLL*, *RP11-529110.4* (*DPCD*) and *FBXW4*. Only C365650 had a larger duplication of 630Kb encompassing all genes from *TLX1* to *FGF8* (Fig.2 and 4).

The proximal breakpoints were spread over an area of more than 100Kb, at least from probe A_16_P39153053 (102876069-102876128bp) to probe A_14_P139489 (102977418-102977470bp). In contrast, for five of the patients the telomeric ends of the duplication were clustered between probes A_16_P02330019 (103468797-103468856bp) and A_16_P02330056 (103498069-103498128bp), except for C365650. In this case the distal breakpoint was flanked by A_14_P123126 (103524624-103524683bp) and A_16_P02330105 (103528589-103528648bp), thus suggesting that at least the last three exons of *FGF8* were also included in the duplication. All affected individuals from a same family shared the same breakpoints (Table 2).

FISH analysis

To confirm the array CGH results, FISH analysis with probes within and flanking the detected aberration was performed. Bacterial artificial chromosomes (BACs) were labeled with red and green dyes, respectively.

Three different combinations of labeled probes were hybridized on slides with spreads from cultured peripheral blood lymphocytes of C2755 and C224256 (Fig.3a-e). In concordance with previous data [9], hybridization with combination of probes within the duplication (BACs RP11-744C22 in red and RP11-619N16 in green) on interphase nuclei from patient C224256 revealed presence of three red and three green signals. Four of the signals, two red and two green respectively, were grouped together. Their linear order red-green-red-green suggested a tandem duplication (Fig.3b). However in the more severely affected brother C2755, FISH with the same probes detected four signals for each BAC, as was expected from the array CGH and Q-PCR results. Six signals, three red and three green, were always clustered but it was not possible to evaluate their relative position (in tandem or inverted orientation) (Fig.3c).

One explanation of the different phenotype in the syndromic SHFM patients could be the more complex character of the observed aberration, representing an insertion instead of simple tandem duplication. Thus the inserted fragment could disrupt a gene(s) in vicinity thus contributing to the additional phenotypic features. To further shed light on the genomic architecture, FISH experiments combining two additional BACs (RP11-108L07 labeled in red and RP11-489E24 in green) flanking the aberration were performed (Fig.3a). Depending on the combination, a string of two/three red or green signals followed by one red or green signal from the probe outside the rearrangement were always present (Fig.3d-e). Thus, the presence of an insertion was excluded.

Finally, to further investigate the suspected somatic mosaicism of the phenotypicaly normal mother (C369371) of C369373 and C369374, a direct FISH analysis with two additional probe combinations was done. Each BAC combination, (1) RP11-774C22 labeled in red and PR11-489E24 labeled in green; and (2) RP11-108L07 labeled in red and RP11-529I10 labeled in green, detected a presence of 30% mosaicism in non cultured peripheral white blood cells (Fig.3f-g).

Q-PCR analysis

Q-PCR assays were additionally carried out to confirm the array CGH result. Two primer pairs, one within BAC RP11-529I10 (Pr1) and another spanning the exonintron boundary of exon 6 of *FGF8* gene (Pr2) were used (Supplementary Table 1). Twelve individuals were included in the analysis. Unfortunately the parents of the original probands (C2755 and C224256) were not available for further study.

In all affected individuals, except one, the Pr1 amplicon showed a 1.5 fold difference, corresponding to the presence of three copies in the analyzed genome. Only for C2755 a fold difference of 2 was detected suggesting the presence of a triplication, which is in correlation with the array CGH and FISH result.

The reported healthy mother (C369371) of C369373 and C369374 consistently showed a 1.24 fold difference. This could be explained if she is a somatic mosaicism carrier, this again in agreement with the 244K Agilent array CGH and FISH results (Fig.4).

All tested individuals except one, had one copy of the FGF8 gene (Pr2). Only C365650 was a carrier of a duplication of at least the last sixth exon of the gene (Fig.4).

| Families | Individuals | Relatives | Phenotype | 1Mb array | 244K array | Q-PCR (Pr1) | Reference |
|----------|-------------|----------------------|------------|--------------|------------------------------|-----------------|--------------|
| | | | | | (genomic position in bp)* | genomic copy No | |
| F1 | C2755 | brother of C224256 | DLDMS | 10q24 | Chr10q 532.77Kb triplication | 4 | Buttiens and |
| | | | | triplication | (102965299-103498069bp) | | Fryns, 1987 |
| | C224256 | sister of C2755 | DLDMS | 10q24 | Chr10q 532.77Kb duplication | 3 | Buttiens and |
| | | | | duplication | (102965299-103498069bp) | | Fryns, 1987 |
| | | | | | | | |
| F2 | C211000 | two healthy siblings | DLDMS | 10q24 | Chr10q 528.72Kb duplication | 3 | Keymolen et |
| | | | | duplication | (102969344-103498069bp) | | al., 2000 |
| | C364835 | mother of C211000 | healthy | na | normal | 2 | This study |
| | C364837 | father of C211000 | healthy | na | normal | 2 | This study |
| | | | | | | | |
| F3 | C369371 | mother of | healthy | na | Chr10q 597.29Kb duplication | 2.5** | This study |
| | | C369373 and C369374 | | | (102900770103498069bp) | | - |
| | C369372 | father of | healthy*** | na | normal | 2 | This study |
| | | C369373 and C369374 | | | | | |
| | C369373 | sister of C369374 | SHFM | na | Chr10q 597.29Kb duplication | 3 | This study |
| | | | | | (102900770-103498069bp) | | - |
| | C369374 | brother of C369373 | DLDMS | 10q24 | Chr10q 597.29Kb duplication | 3 | This study |
| | | | | duplication | (102900770-103498069bp) | | |
| | | | | | | | |
| F4 | C365650 | none | sSHFM | 10q24 | Chr10q 658.43Kb duplication | 3 | This study |
| | | | | duplication | (102870153-103528589bp) | | |
| | C381159 | mother of C365650 | healthy | na | na | 2 | This study |
| | C381158 | father of C365650 | healthy | na | na | 2 | This study |

Table 2. Results of genetic studies.

Legend: SHFM- Split Hand Foot Malformation; sSHFM- syndromic SHFM; DLDMS- Distal limb deficiencies with micrtognathia syndrome; na- not analyzed; *- max size of the aberration; **- carrier of somatic mosaicism; ***- surgically corrected congenital esophageal atresia, micrognathia.

DISCUSSION

Syndromic forms of SHFM are caused by a 10q24 duplication

In this study 10q24 duplications were detected in six patients with Distal limb deficiencies, micrognathia syndrome (DLDMS) or syndromic forms of SHFM. All cases had duplications between 500Kb and 650Kb, including the LBX1, BTRC, POLL, RP11-529110.4 (DPCD) and FBXW4 genes. In one of them (C365650) TLX1 and at least the last three exons of FGF8 gene were also engaged. Thus the size of the aberrations overlapped those of previously published non-syndromic SHFM3 cases with 10q24 rearrangements. However, the affected individuals in this study had a more severe and complex clinical phenotype including specific facial gestalt (4/6 patients), hearing problems (3/6 patients) and kidney dysfunction (3/6 patients). One patient had mental retardation. The limb defects ranged from classical SHFM of hands and feet (2/6 patients) to severe symmetric terminal reduction defects of all extremities (3/6 patients). One patient (C369374) had severe reduction defects of the hands (atypical SHFM) and typical SHFM of feet. Compared to a previous survey [10] the upper limbs of our patients were also more severely affected than the lower, with mainly preaxial involvement and one remaining lateral (fifth) digit ray (4/6 patients).

How does the 10q24 duplication cause syndromic and non-syndromic phenotypes? Until now, there is no explanation how the observed 10q24 genomic aberration causes the phenotype of SHFM. Expression profiling of several candidate genes did not resolve the question [2, 24]. Since these experiments were done using immortalized EBV cell lines, this may not represent the expression of developmental genes during embryogenesis. In addition, *Dactylaplasia* mice were thought to be the corresponding model of SHFM3. It was shown that the phenotypes were caused by two (Dac^{1J}) and Dac^{2J}) highly identical LTR retrotransposon insertions of a type D mouse endogenous provirus element (MusD). The presence of the Dac phenotype was under the control of a homozygous *Mdac* trans-allele, which can completely block the development of skeletal abnormalities. However, even the observed normal skeletal phenotype in heterozygous for Mdac Dac^{2J}-animals, the reduced dactylyn expression was not restored, thus raising questions about the causal effect of *Fbxw4*. Moreover, if the phenotype in SHFM3 patients was the result of increased copies or expression levels of gene(s) lying within the duplication, the corresponding ortholog(s) in Dac mice should also have a disturbed expression. None of Lbx1, Btrc, Poll and Dpcd showed any changes [16, 17, 29]. In addition, several other genes in the vicinity (FGF8, IKKa, WNT8b, SUFU, and NFJB2) also have a role in normal limb development. Therefore, Friedli et al. [15] recently suggested that the genomic aberration can alter the proper balance and interactions between more than one cis-acting regulatory elements and their target genes. Thus, the abnormal phenotype could be the result of disturbed function of several genes and a different extent of the duplication could contribute to the observed phenotypic differences, respectively.

To answer this question, a fine mapping of the duplication size was done using a high resolution oligo-array platform. All syndromic SHFM patients in this study, compared with non-syndromic cases, had a rearrangement encompassing the whole FBXW4 gene at the telomeric end. This extension of the aberration can not explain the phenotypic differences since the individual (C365650) with the largest rearrangement in this group, including at least the last three exons of FGF8 gene, was not more severely affected. Furthermore, published cases with larger abnormalities of chromosome 10q (deletions and/or duplications), including the SHFM3 locus, do not

have any SHFM like defects [28]. All these data indicate that there is no obvious correlation between the size of the 10q24 duplication and the phenotype.

In one patient (C2755) a triplication of 10q24 was detected. He had almost complete reduction of all elements of both forearms and hands, and a more pronounced mental deficit, while his less severely affected sister (C224256) was a carrier of duplication (Table 1, Table 2 and Fig.2-4). Thus the presence of a cryptic triplication instead of a duplication of causative sequences within the SHFM3 locus could be another possible explanation for the observed syndromic phenotype. However, with this resolution of array-CGH platform (Agilent 244K) no similar observations for the other cases in this study were detected. Therefore the triplication itself could contribute to the phenotypic severity in this particular patient but cannot explain the extra-skeletal abnormalities.

We can also not explain how this triplication occurred. There are no flanking low copy repeats, which could promote a recurrent non-allelic homologous recombination. Probably other DNA instability mechanisms like Break-Induced Replication Repair and Fork Stalling and Template Switching are involved [4, 22]. Further research and breakpoint sequencing of more patients are necessary to unravel the genomic mechanisms causing these 10q24 rearrangements.

In 2/4 of our families, there were two affected siblings with healthy parents. Parental samples were available only from one couple (C369373 and C369374) and presence of somatic mosaicism was confirmed in the mother. Her clinical and X-ray examination did not reveal any visible abnormalities. However 3/21 [13 and this study] currently known SHFM3 cases are the result of parental somatic and/or germline mosaicism for the 10q24 microduplication. Thus, at least for some patients, this also could be an explanation for the different extent of observed abnormalities. Genetic counseling of affected families should consider this.

A final possible explanation for the observed phenotypic differences could be the presence of polymorphism(s) (mutations) in causal gene(s) or regulatory motif(s) on the second allele. The skeletal abnormalities observed in our patients are similar to those observed in homozygous conditional Fgf8 knock-out mice. The heterozygous Fgf8 knock-outs are normal but there are other examples where a dominant phenotype in humans is only observed in homozygous mice and could be associated with long range regulator effects, e.g. *Shh* and holoprosencephaly [8, 21].

Additional support for a possible role of FGF8 in the observed skeletal and extraskeletal defects is given by transgenic studies looking for regulatory sequences downstream of Fgf8 (Fig.4) [5, 20, 33]. Intriguingly, in these transgenic animals the studied highly conserved elements can specifically drive the expression of the reporter gene within the AER, otic placode, nephrogenic cord, maxillary and mandibular arches. Some of these regulatory sequences can also partially rescue the mouse phenotype of Fgf8 double null allele. The mutant mice survive until birth and die shortly thereafter demonstrating an abnormal facial development with maxillary and mandibular hypoplasia, an abnormal "open eye" phenotype, and an inappropriate limb development [5]. This expression profile, cranio-facial and limb dysmorphism cover all the observed anomalies in our patients– maxillary hypoplasia, micrognathia, hearing problems, renal hypoplasia/dysfunction and truncation limb defects. Therefore looking for polymorphisms/mutations in *FGF8* and its regulatory sequences in the future could extend our knowledge as to how this 10q duplication causes abnormal phenotypes.

In summary, this study demonstrates that 10q24.31q24.32 microduplications also cause syndromic forms of SHFM. There is high variability in the observed skeletal

anomalies and Distal limb deficiencies with micrognathia syndrome (MIM 246560) represents the most severe end of SHFM3 spectrum. These findings strongly support the importance of array CGH analysis in entities, where the underlined etiology and/or the real extent of the clinical phenotype are not well defined. For example, Patterson-Stevenson-Fontaine syndrome (PSFS) is a condition combining partially the facial gestalt and the skeletal anomalies of DLDMS, syndromic/nonsyndromic SHFM3 patients and SHFM1 cases. The observed intra-familial variability of the PSFS is similar to the one detected in DLDMS and familial SHFM3 [34]. Recently, Bigo et al. [6] detected a 7q21.3 microdeletion, containing 3 genes- DLX5, DLX6 and DSS1, in the originally described by Fontaine et al. family [14]. However, no similar chromosomal aberration was found in the second PSFS family published by Patterson and Stevenson [26]. Further research needs to unravel to what extent these conditions, as well as other rare forms of acrofacial dysostoses share common etiology and/or pathways. Until now there is no reliable explanation how the 10q24 genomic aberration causes an abnormal limb development or why some patients have extraskeletal anomalies. It is also difficult to apply one common mechanism to explain the phenotype both in humans and Dactylaplasia mice. In conclusion, the real contribution of FBXW4, FGF8 and other genes within or in the vicinity of the duplication associated with the observed phenotypic spectrum still remains to be discovered.

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FIGURE LEGENDS

Figure 1. Clinical phenotypes of case C2755, C369374 and C365650. Written consent was given to the authors for publication of the patient's images in print and online: a-c) C2755 with LDMS originally published by Buttiens and Fryns [6]. All four limbs are symmetrically affected. There is marked facial asymmetry with pronounced micrognathia, maxillary hypoplasia and short stature; d-g) The facial gestalt of C369374 is similar to that of the affected siblings in Family 1- maxillary hypoplasia, micrognathia and low set ears. There are symmetrical terminal reduction defects of both hands with one ulnar finger remaining. An appendage like mass on the left hand is medially linked by a skin bridge to the palm. A large medial gap separates the two digits of both feet; h-j) Hand and foot radiographs of C369374 demonstrate symmetrical reduction of autopod elements of all four limbs. There are only four metacarpals and one ulnar (fifth) digit with three phalanges of both hands. In addition, an accessory short tubular bone pointing to the fifth metacarpo-phalangeal joint and one not very well defined ossification centre of bone remnant are present. Both feet have a classical SHFM phenotype with three metatarsal bones and two digits, each with phalanges. The last two metatarsals are fused at their distal ends and a big gap separates them from the first metatarsal. There is no ossification of carpal bones at that age and two tarsal bones are present; k and l) classic four limb SHFM phenotype of patient C365650.

Figure 2. Agilent 244K array result of all six patients carrying a 10q duplication, C369371- the suspected of somatic mosaicism mother of C369373 and C369374, and the phenotypicaly normal father C369372 of the same siblings. For the exact position of the breakpoints and the size of the aberration in all four families see the text and Table 2. The severity of the phenotype corresponds to the colour marking the aberration- from grayish-blue as the most severe to light-blue as the less severe observed phenotype.

Figure 3. a) Five BACs, three within and two flanking the duplication, were labeled in red and green as shown. The dashed bar represents the aberration and the colored circles the corresponding FISH probes. Below each individual experiment in (b), (c), (d), (e), (f) and (g) a similar schematic graph shows the position and colour of the used FISH probes; b) Interphase FISH analysis with probes RP11-774C22 (red) and RP11-619N16 (green), both within the 10q24 duplication, revealed the presence of a tandem duplication in C224256; c) The same combination of BACs demonstrated the presence of four hybridization signals for both probes in lymphocyte nuclei of C2755, her more severely affected brother. Three red and three green signals were always brought together, suggesting that they share one allele; d) Interphase FISH with combination of BAC RP11-489E24 (lying distally to the aberration) and BAC RP11-774C22 (within the rearrangement) showed the presence of four red signals and two green signal in C2755. A string of three red and one green and separately paired red and green signals were present in each screened nucleus; e) In the same patient (C2755), a similar result was obtained using a combination of BAC RP11-108L07 (proximal to the duplication/triplication) and BAC RP11-619N16 lying within the aberration; f-g) Direct FISH with two different probe combinations: (1) RP11-774C22 labeled in red and PR11-489E24 labeled in green, and (2) RP11-108L07 labeled in red and RP11-529I10 labeled in green, demonstrates 30% somatic mosaicism in white blood cells of individual C369371.

Figure 4. Organization of 10q24 duplication locus (not to scale): a) The brown line represents the genomic organization of the 10q24 locus duplicated in nonsyndromic and syndromic SHFM patients. Filled brown bars correspond to the genes and arrows indicate the 5' end of each of them. The black line above highlights some parts of the aberration- BTRC, FBXW4 and the last two exons of FGF8, as well as the area between the last two genes. Vertical lines demarcate the exons of each pointed gene. The mutations in Dactylaplasia mice Dac^{1J} and Dac^{2J} are indicated with red triangles above the line (Kano et al., 2007). Blue and purple squares highlight the positions of Pr1 and Pr2, also shown in (b). Red triangles above and green triangles below the line demonstrate the position of regulatory sequences in mice and zebrafish, proven to drive the expression of reporter genes within the AER and in developing limbs (for CR3 see Beermann et al., 2006; for #667 and #508 see Kikuta et al., 2007; and for e326 and e511 see VISTA Enhancer Browser http://enhancer.lbl.gov/frnt_page.shtml and Visel et al., 2007); b) Red, green, blue and purple bars and squares present the position of BACs and primer pairs Pr1, and Pr2 used for FISH, and Q-PCR. They are aligned to the map shown in (a) but for their exact position see the text and table 3; c) Black lines F1, F2, F3 and F4 show the family specific size of the 10q24 duplication with respect to the gene positions represented with brown bars in (a). For exact breakpoint positions see the text and Table 2. The last line named SHFM3 summarizes the duplication sizes of previously published cases. The dotted part of the line represents the different breakpoint positions as described (de Mollerat et al., 2003; Kano et al., 2005 and Lyle et al., 2006); d) Q-PCR with primer pair Pr1 confirmed the presence of one additional copy in all affected individuals, except one. Only C2755 had fold difference of 2 using the same primers. This corresponds to the presence of a triplication in this individual. In addition, the healthy mother C369371 of C369373 and C369374 had fold difference of 1.25, which in addition to the FISH and array CGH data confirms that she is a carrier of somatic mosaicism. Q-PCR using Pr2 primer pair demonstrated a presence of one additional copy only in patient C365650.

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