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Deletions of exons with regulatory activity at the *DYNC111* locus are associated with split-hand/split-foot malformation: array CGH screening of 134 unrelated families

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Abstract

Background: A growing number of non-coding regulatory mutations are being identified in congenital disease. Very recently also some exons of protein coding genes have been identified to act as tissue specific enhancer elements and were therefore termed exonic enhancers or “eExons”.

Methods: We screened a cohort of 134 unrelated families with split-hand/split-foot malformation (SHFM) with high resolution array CGH for CNVs with regulatory potential.

Results: In three families with an autosomal dominant non-syndromic SHFM phenotype we detected microdeletions encompassing the exonic enhancer (eExons) 15 and 17 of *DYNC111*. In a fourth family, who had hearing loss in addition to SHFM, we found a larger deletion of 510 kb including the eExons of *DYNC111* and, in addition, the human brain enhancer hs1642. Exons 15 and 17 of *DYNC111* are known to act as tissue specific limb enhancers of *DLX5/6*, two genes that have been shown to be associated with SHFM in mice. In our cohort of 134 unrelated families with SHFM, deletions of the eExons of *DYNC111* account for approximately 3% of the cases, while 17p13.3 duplications were identified in 13% of the families, 10q24 duplications in 12%, and *TP63* mutations were detected in 4%.

Conclusions: We reduce the minimal critical region for SHFM1 to 78 kb. Hearing loss, however, appears to be associated with deletions of a more telomeric region encompassing the brain enhancer element hs1642. Thus, SHFM1 as well as hearing loss at the same locus are caused by deletion of regulatory elements. Deletions of the exons with regulatory potential of *DYNC111* are an example of the emerging role of exonic enhancer elements and their implications in congenital malformation syndromes.

Keywords: SHFM, *DLX5/6*, *DYNC111*, Regulatory Mutations, eExons

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Background

Split-hand/split-foot malformation (SHFM; OMIM 183600), also referred to as ectrodactyly, is a congenital limb malformation, characterized by a claw-like appearance of the distal portion of the upper and lower limbs with profound median clefts of hands and feet and missing or malformed central fingers [1]. This disorder is clinically heterogeneous, comprising both isolated as well as syndromic forms [2]. SHFM is typically inherited as an autosomal dominant trait with incomplete penetrance, variable expressivity and segregation distortion [3]. However, autosomal recessive inheritance has been reported [4-6]. The pathogenesis of SHFM has been studied in various mouse models. A failure to maintain signalling from the median apical ectodermal ridge (AER) has been shown to be a main pathogenic mechanism [7-9]. Without this signalling, cells of the underlying progress zone stop proliferation and differentiation which in turn results in defects of the central rays. The time point and extent of this signalling deficit determines the phenotype. Early and more extensive defects will result in more severe deficiencies and vice versa.

At least 5 different loci have been associated with non-syndromic SHFM. The most common form, which may also feature long bone (tibial hemimelia) deficiency (SHFLD) is caused by duplications involving the gene *BHLHA9* [10] (SHFLD3; MIM 612576). SHFM3 (OMIM 600095) is associated with the duplication of chromosome 10q24-q25, which encompasses the *Dactylin* gene (*FBXW4*) [11]. SHFM4 (OMIM 605289) is caused by mutations in *TP63* on 3q27 [12]. The gene appears to be essential for ectodermal development and a deficiency affects function and maintenance of the AER. SHFM5 (OMIM 606708) maps to 2q31. Although the disease gene has not been identified for this locus yet, a contribution of *HOXD* gene cluster has been suggested for SHFM5 [13]. SHFM1 (OMIM 183600) maps to 7q21.2-q21.3 including the genes *DLX5*, *DLX6* and *SHFM1*.

SHFM1 is an autosomal dominant trait with reduced penetrance and variable expression ranging from mild defects of a single limb to severe abnormalities of all four extremities [14]. SHFM1 is associated with mental retardation in 33% of patients, craniofacial malformations in more than 35% of patients, and deafness in 35% of patients (SHFM1D; OMIM 220600) [15]. Physical mapping of the SHFM1 locus on chromosome 7 defined a critical interval of about 1.5 Mb that encompasses *SHFM1* (deleted in the split-hand/split-foot 1 region), *Dlx5* and *Dlx6* (distal less-related homeobox gene 5 and 6), of which only *Dlx5/6* have been shown to play a role in early limb development [16,17]. The *Dlx5* and *Dlx6* are expressed in the AER of the embryonic limb buds, craniofacial prominence, otic vesicle, and in the brain.

Dlx5 deficient mice do not show any limb defects [18]. However, disruption of both *Dlx5* and *Dlx6* in mice leads to ectrodactyly [7]. So far a single consanguineous family with severe SHFM1 phenotype and hearing impairment was demonstrated to carry a homozygous missense mutation in *DLX5* [19]. Furthermore, Wang et al. identified a novel heterozygous missense mutation in exon 3 of *DLX5* in a Chinese family with autosomal dominant SHFM1 [20].

Recently, exons 15 and 17 of *DYNC111* (dynein cytoplasmic 1 intermediate chain 1) were identified as “coding” limb enhancers of *Dlx5/6* in mice and zebrafish [21]. The two coding exons act as “exonic enhancers” or “eExons” and were shown to physically interact with the promoters of *Dlx5/6* in mice using chromosome conformation capture analysis [21]. *Dync111* itself is not expressed during limb development. In addition, an enhancer element within the intronic region of *SLC25A13* (solute carrier family 25 member 13) was shown to regulate tissue specific *Dlx5/6* expression in the otic vesicle, forebrain, branchial arch and limb bud mesenchyme. The deletion of *Slc25a13* in mice is not associated with a SHFM phenotype [22-24].

Here we report on three unrelated families with overlapping deletions of the *cis*-regulatory eExons 15 and 17 of *DYNC111* located over 900 kb centromeric to their target genes *DLX5/6* on chromosome 7q21.3. In a fourth family presenting with SHFM and hearing loss we detected a 510 kb deletion including the eExons 15 and 17 of *DYNC111* but also *SLC25A13*, *C7orf76*, and several brain enhancers. In our cohort of 134 unrelated families with SHFM and SHFLD deletions of the eExons of *DYNC111* on chromosome 7q21.3 account for approximately 3% of the cases. These data have a direct impact on SHFM1 diagnostics and further highlight the role of regulatory mutations and structural variations in congenital disease.

Material and methods

Patients

In a cohort of 134 unrelated families with SHFM we excluded the known causes of SHFM and SHFLD i.e. 17p13.3 duplications with ectrodactyly and tibia hemimelia [25], 10q24 duplications and mutations in *TP63* with the clinical manifestation of ectrodactyly without tibial involvement by quantitative real-time PCR (qPCR) and Sanger sequencing, respectively [25]. Balanced rearrangements were not excluded. The remaining families were used to screen for CNVs using high resolution (1 M Agilent) array CGH. Written informed consent was obtained from all individuals studied, or their parents or guardians in the case of children under 16, to participate in the study and for publication of this manuscript and any accompanying images. The

study was approved by the Charité Universitätsmedizin Berlin ethics committee as well as by the Institutional Review Board of Poznan University of Medical Sciences.

Microarray-based comparative genomic hybridization (array CGH)

All experiments were done with genomic DNA extracted from blood samples. Array CGH for family 1, 3, and 4 was carried out using a whole genome 1 M oligonucleotide array (Agilent, Santa Clara, CA). 1 M arrays were analyzed by Feature Extraction v9.5.3.1 and CGH Analytics v3.4.40 software or Cytogenomics v2.5.8.11, respectively (Agilent, Santa Clara, CA). Family 2 was tested on *Roche NimbleGen* platform with use of 1.4 M oligo arrays according to standard protocols provided by the manufacturer. Analysis settings: aberration algorithm: ADM-2; threshold: 6.0; window size: 0.2 Mb; filter: 5probes, log2ratio = 0.29. The genomic profile was visualized by the SignalMap software (SignalMap v1.9.0.03, NimbleGen Systems Inc.). Data were submitted to the DECIPHER database (<http://decipher.sanger.ac.uk>); accession numbers: BER284939, BER284938, BER284937, and BER285016.

Quantitative real-time PCR (qPCR)

qPCR was performed as described previously [26,27] using genomic DNA of the index patients and further family members to confirm the deletions and to show segregation with the phenotype. Primer sequences are given in Additional file 1: Table S1.

Breakpoint analysis

The exact determination of the deletion size was done by breakpoint spanning PCR following the qPCR analyses and sequencing of the junction fragment (primer sequences and positions in Additional file 1: Table S1 and Additional file 1: Figure S1).

PCR was performed in a total volume of 20 µl with 40 ng genomic DNA as template, 2 µl 10× PCR buffer, 0.6 µl dNTP mix (10 mM), 0.5 µl primer (10 pMol/µl), 0.6 µl MgCl₂ (50 mM), 0.2 µl Taq polymerase (Rapidozym, Germany). PCR conditions are available upon request. The PCR products were purified by enzymatic treatment (Exonuclease I, NEB; Shrimp Alkaline Phosphatase, Roche Diagnostics). For the sequencing of the PCR products the BigDye v3.1 (Applied Biosystems) sequencing kit was used. PCR products were analyzed by

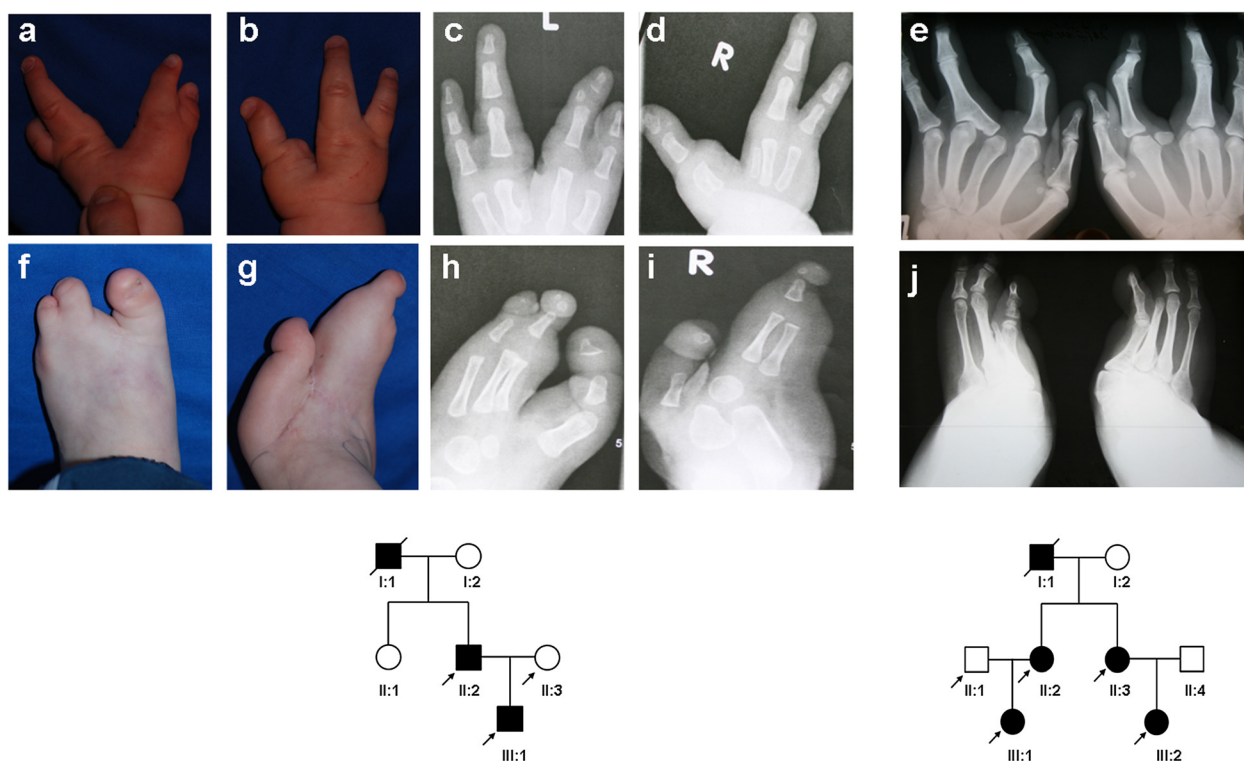


Figure 1 Limb phenotypes and pedigrees of families 1 and 2. Family 1 is a non-consanguineous family from Poland with three affected individuals. All affected individuals showed split hands and feet in all four extremities. Hands (a-d) and feet (f-i) of the affected one-year-old son. No long bone involvement or other bone phenotype was reported. There was no history of hearing impairment in the family. Neurological and motor development was normal. Family 2 is a non-consanguineous German family with five affected individuals. All affected individuals showed SHFM in both hands and feet and severe hearing loss. The hands (e) and feet (j) of a 69-year-old affected female. No long bone involvement or other bone phenotype was reported. Neurological and motor development was normal in all family members.

capillary automat ABI3730 (Applied Biosystems). The sequencing results were processed by DNA-STAR software (DNA-Star).

Results

Phenotypic analysis

We investigated a total of 16 affected from 4 unrelated families.

Family 1 is a non-consanguineous family from Poland with three affected individuals (grandfather, father and son), all showing the classical SHFM phenotype in all four extremities. Figure 1 (a-d & f-i) shows the hand and feet of the affected one-year-old son. Neither long bone involvement nor craniofacial abnormalities were noted. There was no history of hearing impairment in the family. Neurological and motor development was normal.

Family 2 is a non-consanguineous German family with five affected individuals (a grandfather, two affected sisters

and their two daughters). Four affected individuals showed SHFM in both hands and feet and, in addition, severe hearing loss. One female showed SHFM in both hands but only one foot and hearing loss. Figure 1 (e and j) shows the hands and feet of a 69-year-old affected female. Neither long bone involvement nor craniofacial abnormalities were noted. Neurological and motor development was normal in all family members.

Family 3 is a non-consanguineous family of Polish origin with six individuals from three generations all affected by SHFM. All patients presented with variable limb malformations, ranging from a severe cleft in all limbs (see index in Figure 2a, b) to milder defects, such as absent or hypoplastic central digit (aunt of the index in Figure 2c). None of the patients from family 3 manifested long bone deficiency or hypoplasia. Psychomotor development and hearing of all affected family members was normal.

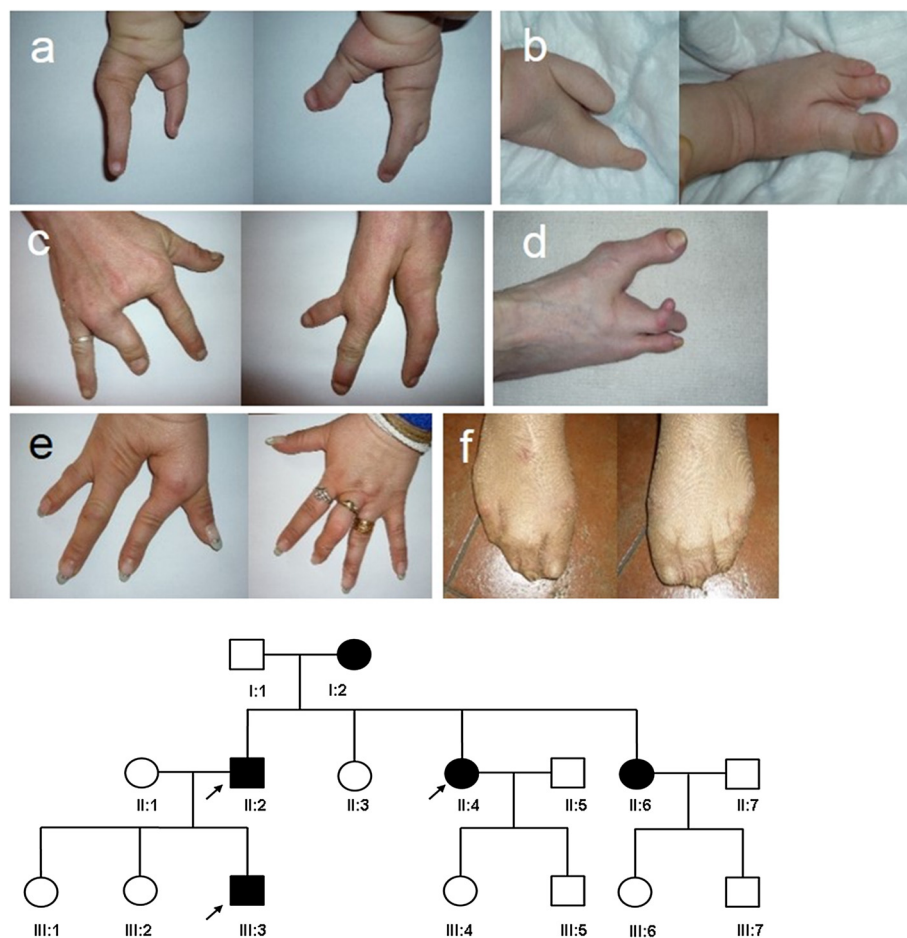


Figure 2 Limb phenotypes and pedigree of family 3. Family 3 is a non-consanguineous family from Poland with six affected individuals (grandmother, two affected sisters and their brother, and two male offspring of the brother and one of the sisters). All patients showed SHFM in all for limbs with no long bone involvement. Hearing loss was not reported in this family. The proband shows bilateral SHFM of the hands (a) and feet (b). The father of the proband shows central ray deficiency of the hands (c) and right foot (d). The aunt of the proband presents milder phenotype on the left hand (e) and feet (f).

Family 4 is a non-consanguineous German with two affected sisters (monozygotic twins) showing a SHFM phenotype in the hands only. Neither long bone involvement nor craniofacial abnormalities were noted. There was no history of hearing impairment in the family. Neurological and motor development was normal.

In our cohort of patients with deletions of the eExons of *DYNC111* the penetrance was high. However, due to the small sample size future studies are needed to determine the penetrance of *DYNC111* deletions.

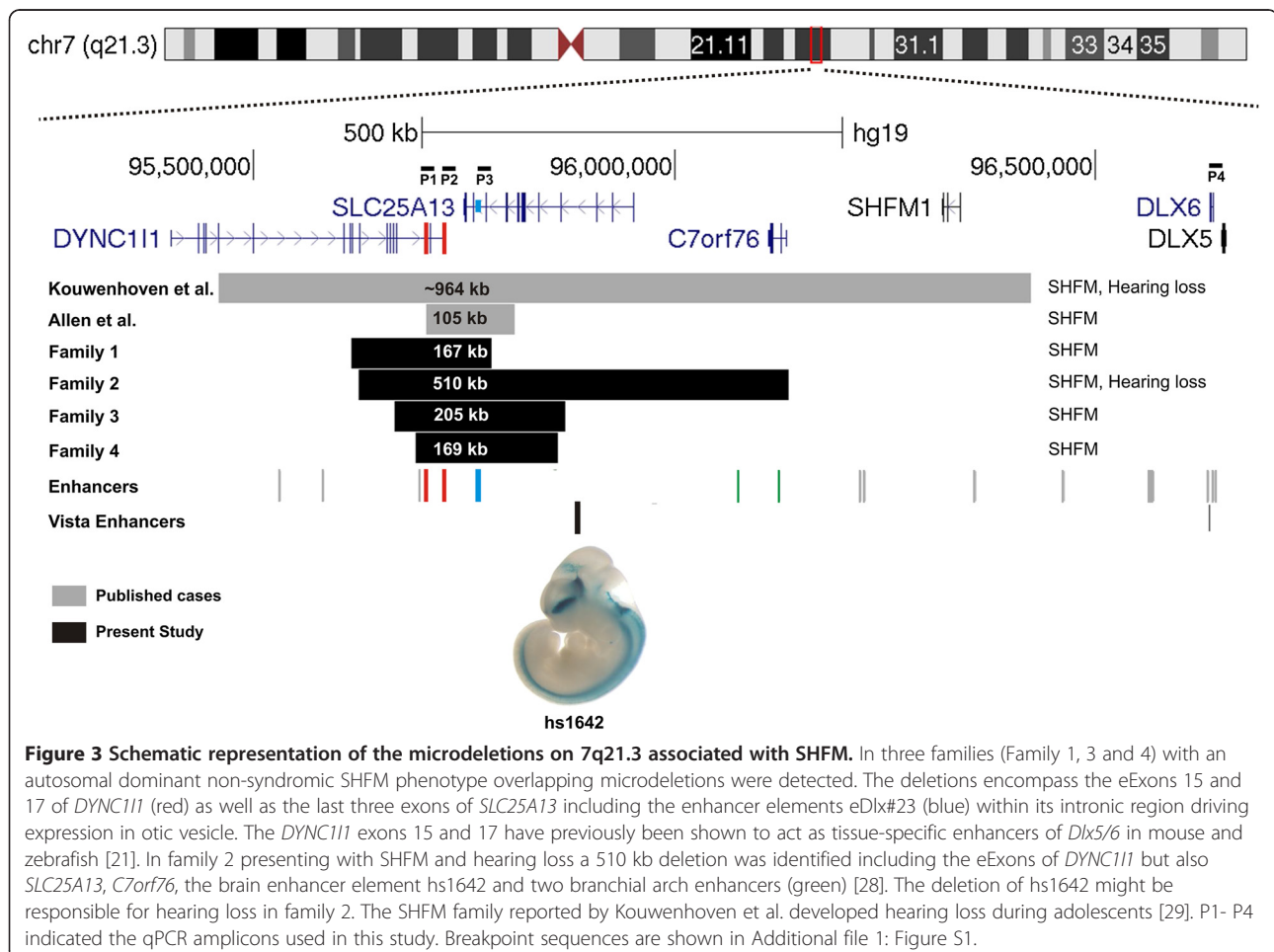
Deletions of the eExons 15 and 17 of *DYNC111* are associated with SHFM1

We identified three unrelated families with overlapping deletions of the *cis*-regulatory eExons of *DYNC111* over 900 kb centromeric to *DLX5/6* with a split-hand/split foot phenotype (Figure 3). In families 1, 3, and 4 we detected overlapping microdeletions of 167 kb, 205 kb and 167 kb, respectively. The deletions encompass the eExons 15 and 17 of *DYNC111* as well as the last three exons of *SLC25A13* including the enhancer elements eDlx#23 within its intronic region (Figure 3). The latter

has been shown to drive expression in the otic vesicle, forebrain, branchial arch and limb bud mesenchyme. The *DYNC111* exons 15 and 17 have previously been shown to act as tissue-specific enhancers of *Dlx5/6* in mouse and zebrafish [21]. The deletions are therefore likely to result in tissue-specific loss of function of *DLX5/6* in the developing hand or foot causing SHFM.

In family 2 presenting with SHFM and hearing loss we detected a 510 kb deletion encompassing the eExons of *DYNC111* but also *SLC25A13*, *C7orf76* and the human brain enhancer element hs1642 (Figure 3).

The array CGH results were confirmed by qPCR in the index patients. Analysis of further affected and unaffected family members of all 4 families showed that the microdeletions segregate with the phenotype, i.e. unaffected family members had a normal copy number of the region. The qPCR data showed only one copy of the analyzed amplicons, i.e. a deletion, in all affected individuals. Similar deletions were not observed in 200 control DNA samples and are were not present in the Database of Genomic Variants (DGV) (<http://dgv.tcag.ca/dgv/app/home>).



Breakpoint analysis

In order to determine the exact breakpoints of the microdeletions and to exclude translocations or inversions, junction fragments that included the telomeric and centromeric breakpoints were PCR amplified and subsequently sequenced in all affected individuals. Deletion breakpoints were detectable only in the affected, but not in the unaffected individuals. In family 1 we detected a 167 kb deletion on 7q21.3 (genomic position chr7:95,615,187-95,783,313). In family 2 we detected a 510 kb deletion on 7q21.3 (genomic position chr7:95,624,825-96,135,521). In family 3 a 205 kb deletion on 7q21.3 (genomic position chr7:95,667,046-95,872,044) was detected (Table 1). Breakpoint spanning sequences are shown in Additional file 1: Figure S1. In family 4 we detected a 169 kb deletion on 7q21.3 (maximum genomic position chr7:95,693,341-95,862,369). For family 4, it was not possible to obtain additional DNA to performer breakpoints sequencing. All positions are given in hg19.

Deletions of eExons of *DYNC111* account for approximately 3% of SHFM/SHFLD cases

In our cohort of 134 unrelated families with SHFM and SHFLD the following molecular analyses were performed: qPCR for 17p13.3 duplications, qPCR for 10q24 duplications, qPCR for deletions of the eExons 15 and 17 of *DYNC111* on 7q21.3, and mutational analysis for *TP63*. *WNT10B* mutation analysis was only performed in seven consanguineous families. Balanced rearrangements were not excluded.

In our cohort, 17p13.3 duplications were identified in 13% (18/134) of the families, 10q24 duplications in 12% (16/134), *TP63* mutations were detected in 4% (6/134), and deletions of the eExons of *DYNC111* in 3% (3/134) (Figure 4). Thus, in 68% of the families with SHFM and SHFLD no molecular diagnosis was possible.

Conclusions

Congenital malformations represent a major medical problem with a prevalence of 2-6% in humans [30]. Elucidation of the genetic basis of this heterogeneous group of disorders has been tremendously successful

Table 1 Microdeletions of various sizes encompassing the exonic enhancers of *DLX5/6* located in exon 15 and 17 of *DYNC111* are associated with SHFM1

| Family | Genomic Location (hg19) | Size of Deletion [kb] | Phenotype |
|--------|-----------------------------|-----------------------|--------------------|
| 1 | chr7:95,615,187-95,783,313 | 167 | SHFM |
| 2 | chr7:95,624,825-96,135,521 | 510 | SHFM, Hearing loss |
| 3 | chr7: 95,667,046-95,872,044 | 205 | SHFM |
| 4 | chr7:95,693,341-95,862,369 | 169 | SHFM |

SHFM: Split hand foot malformation.

over the last decades by the interpretation of disease related variations in the 1.5% of protein coding DNA and the identification of variants that result in the disruption of specific gene function [31]. However, the growing number of regulatory mutations mainly located in the non-coding DNA affecting *cis*-regulatory elements highlights the clinical importance of long range gene regulation in congenital disease [32,33]. Very recently also some exons of protein coding genes have been identified to act as tissue specific enhancer elements and were therefore termed exonic enhancers or “eExons” [34].

In this study, we report on four unrelated families with overlapping deletions of the *cis*-regulatory eExons 15 and 17 of *DYNC111* that act as tissue specific limb enhancers of *DLX5/6* which is located over 900 kb telomeric on chromosome 7q21.3. In three families with an autosomal dominant non-syndromic SHFM phenotype affecting all four limbs we detected overlapping microdeletions of 167 kb, 169 kb and 205 kb. The deletions encompass the two limb specific eExons 15 and 17 of *DYNC111* as well as the last three exons of the *SLC25A13* gene. In family 2 presenting with SHFM and hearing loss we detected a 510 kb deletion including the eExons 15 and 17 of *DYNC111* but also *SLC25A13*, *C7orf76*, two branchial arch enhancers and the human brain enhancer element hs1642. All four deletions encompass the eExons of *DYNC111*. Interestingly, the enhancer element eDlx#23 that was shown to drive reporter expression in otic vesicle, forebrain, branchial arch and limb bud mesenchyme was also deleted in all three families, but only family 2 manifested hearing loss (Figure 3) [21]. Lango Allen et al. reported on a family with SHFM but without hearing loss carrying a similar 106 kb deletion also including the eExons of *DYNC111* and the otic vesicle enhancer elements eDlx#23 [35]. These data suggest that the loss of otic vesicle enhancer eDlx#23 is not pathogenic in these patients. Rather, it is more likely that hearing loss in family 2 results from the deletion of the telomeric 264 kb region encompassing two branchial arch enhancers, the human enhancer element hs1642, and *C7orf76* (Figure 3) [21,28]. The human enhancer element hs1642 shows a tissue specific reporter expression in the forebrain, the hindbrain (rhombencephalon), and the neural tube (Figure 3) [28]. This region also includes the auditory forebrain and might therefore be a potential candidate for hearing loss [36].

Another large deletion of over 900 kb was described by Kouwenhoven et al. in an individual that was affected with SHFM and later also developed hearing loss. In this study, they showed that p63 binds to an enhancer element that is located between *SHFM* and *DLX5/6* [29]. In another recent study Restelli et al. report that the fibroblast growth factor 8 (*FGF8*) locus is a downstream target of *DLX5*. Furthermore they show that *DLX5*, p63,

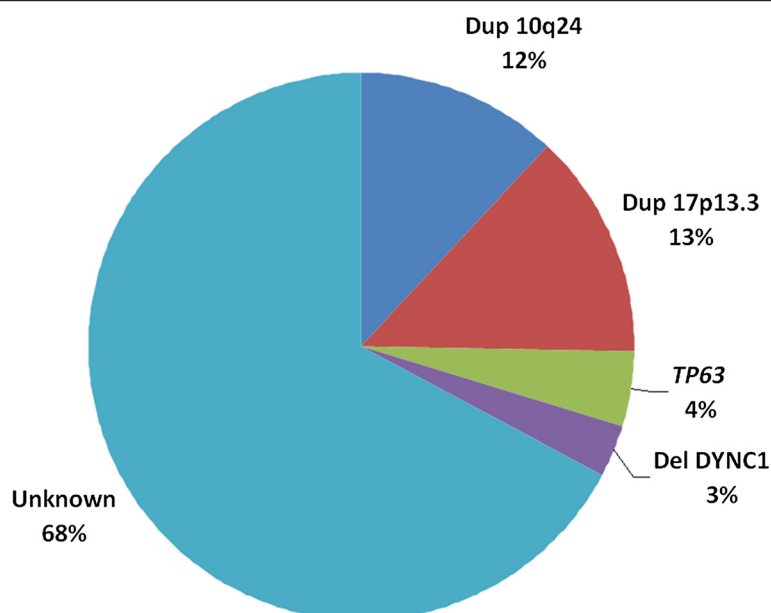


Figure 4 Deletions of eExons of *DYNC111* account for approximately 3% of SHFM/SHFLD cases: In our cohort of SHFM/SHFLD cases, 17p13.3 duplications were identified in 13% (18/134) of the families, 10q24 duplications in 12% (16/134), *TP63* mutations were detected in 4% (6/134), and deletions of the eExons of *DYNC111* in 3% (3/134). *WNT10B* mutation analysis was negative in all seven consanguineous families investigated. In the majority of families with SHFM and SHFLD (68%) no molecular diagnosis could be made.

Pin1 and FGF8 are essential for AER stratification and normal patterning and skeletal morphogenesis of the limb buds [37]. Several other translocations and inversions at the SHFM1 locus have been described that separate *DLX5/6* from their regulators located between *DYNC111* and *DLX5/6* [21]. Depending on the location of the breakpoint a genotype-phenotype correlation seems now feasible. Our results confirm that deletions of the *cis*-regulatory eExons 15 and 17 of *DYNC111* are associated with split-hand/split foot and we reduce the SHFM1 minimal critical region to 78 kb (Figure 3). Hearing loss, however, seems to be associated with deletions of a more telomeric 264 kb region encompassing the brain enhancer element hs1642 (Figure 3). Craniofacial defects seem to result from breakpoints located in the region closest to *DLX5/6* [38].

In order to determine the clinical relevance and prevalence of deletions of the eExons of *DYNC111* we screened our cohort of SHFM and SHFLD cases. In our cohort of 134 unrelated families with SHFM and SHFLD, deletions of the eExons of *DYNC111* on chromosome 7q21.3 account for approximately 3% of the cases. 17p13.3 duplications mainly associate with ectrodactyly and tibia hemimelia [25], while mutations in *TP63* are mainly associated with ectrodactyly without tibial involvement. 10q24 duplications show the highest phenotypical variability ranging from single digit shortening to absence of hands and feet. The introduction of whole exome sequencing into clinical diagnostics will reveal the role of mutations in *DLX5* and

WNT10B in SHFM. Our data have a direct impact on diagnostics for SHFM since they further highlight the importance of array-CGH as a diagnostic tool for SHFM/SHFLD and to detect regulatory mutations [39]. Furthermore the deletions of the eExons of *DYNC111* are an example of the emerging role of exonic enhancer elements and their role in congenital malformations.

Additional file

Additional file 1 Figure S1. Confirmation of microdeletions on 7q21.3 by breakpoint spanning PCRs in family 1-3. **Table S1:** qPCR primers and sequencing primers for breakpoint detection in family 1-3. (DOC 286 kb)

Competing interests

The authors declare no competing interests.

Authors' contributions

Patient recruitment and phenotyping: MS, AJ, ASS, RF, SCD, WH, RH, BOJ, SM, and EK. Array-CGH experiments and analysis: NT, AJ, RF, MS, SM and EK. All the authors contributed in writing and reviewing the manuscript. All authors read and approved the final manuscript.

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