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Original Article

Microduplications encompassing the Sonic hedgehog limb enhancer ZRS are associated with Haas-type polysyndactyly and Laurin-Sandrow syndrome

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Laurin-Sandrow syndrome (LSS) is a rare autosomal dominant disorder characterized by polysyndactyly of hands and/or feet, mirror image duplication of the feet, nasal defects, and loss of identity between fibula and tibia. The genetic basis of LSS is currently unknown. LSS shows phenotypic overlap with Haas-type polysyndactyly (HTS) regarding the digital phenotype. Here we report on five unrelated families with overlapping microduplications encompassing the *Sonic hedgehog (SHH)* limb enhancer ZPA regulatory sequence (ZRS) on chromosome 7q36. Clinically, the patients show polysyndactyly phenotypes and various types of lower limb malformations ranging from syndactyly to mirror image polydactyly with duplications of the fibulae. We show that larger duplications of the ZRS region (>80 kb) are associated with HTS, whereas smaller duplications (<80 kb) result in the LSS phenotype. On the basis of our data, the latter can be clearly distinguished from HTS by the presence of mirror image polysyndactyly of the feet with duplication of the fibula. Our results expand the clinical phenotype of the ZRS-associated syndromes and suggest that smaller duplications (<80 kb) are associated with a more severe phenotype. In addition, we show that these small microduplications within the ZRS region are the underlying genetic cause of Laurin-Sandrow syndrome.

Conflict of interest

The authors declare no conflict of interest.

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Sonic hedgehog (SHH) (MIM 600725) is a member of the hedgehog family and an important regulator during early embryogenesis. During limb development SHH determines the anterior–posterior axis by a morphogenetic gradient and is expressed in the posterior region of the limb bud (Zone of Polarizing Activity, ZPA) (1). The *cis*-regulatory element responsible for controlling expression of *Shh* in the posterior part of the limb was identified by studying the genetic bases of preaxial polydactyly (PPD) in humans, mice, and cats (2). These studies showed that normal *Shh* expression in the ZPA is driven by an upstream enhancer which became known as the ZPA regulatory sequence (ZRS) (1–3). This element is highly conserved in all vertebrates with limb appendages and consists of an 800 bp enhancer sequence located within intron 5 of *Lmbr1* (MIM 605522) around 1 Mb upstream of its target gene *Shh*. It represents one of the prime examples of long-range gene regulation and has been studied intensively in mice and men (1).

In mice, point mutations within the ZRS results in ectopic expression of *Shh* at the anterior margin of the limb (in addition to the normal posterior expression domain). This 'double' dose of hedgehog signal from two sides causes polydactyly as shown in the mouse mutants hemimelic extra toes (*Hx*), and Sasquatch (*Ssq*). Subsequently, several point mutations in humans in the ZRS, as well as microduplications encompassing the human ZRS region have been described to be associated with various limb phenotypes including triphalangeal thumb-polysyndactyly syndrome (TPTPS, MIM 174500), Haas-type polysyndactyly (HTS), also called syndactyly type IV (SD4, MIM 186200), and Werner mesomelic syndrome (WMS, MIM 188770) (2, 4–6). Wieczorek et al. (2010) introduced a classification of the 'ZRS-associated syndromes': type Ia is associated with point mutations in various positions of the ZRS resulting in triphalangeal thumb polydactyly exclusively affecting the hands. Type Ib includes point mutations at position 404 of ZRS associated with triphalangeal thumb polydactyly with hypoplastic tibia. Type II represents the group of ZRS duplications causing various complex polysyndactylies and triphalangeal thumb phenotypes (5).

The Laurin-Sandrow syndrome (LSS; MIM 135750) is a rare autosomal dominant disorder characterized by polysyndactyly of hands and feet, that have been classified as mirror image duplications, nasal defects (hypoplastic alae nasi, short columella), in connection with absent patella and loss of identity between fibula and tibia, i.e. duplicated fibula (7, 8). The term mirror image duplication refers to a complete duplication of all digits with one or two thumb-like structures. Additional clinical features have been described such as intellectual disability and brachymesophalangy of toes, indicating that LSS has a variable clinical presentation (9). The genetic cause of LSS is currently not known but an X-linked inheritance can be excluded because of male-to-male transmission (10).

Here we report five unrelated families with overlapping microduplications of *SHH* limb enhancer ZRS on chromosome 7q36. We show that small microduplications within the ZRS region (<80 kb) are the underlying genetic cause of Laurin-Sandrow syndrome and that larger duplications result in less severe phenotypes.

Material and methods

Patients

We have examined five families with partially overlapping polysyndactyly phenotypes (Figs. 1 and 2). Families 1 and 2 presented with the clinical sings of Haas-type polysyndactyly featuring severe cup-shaped hands with polydactyly (Fig. 1). Family 1 is a non-consanguineous family from Peru. The affected boy showed a complete syndactyly of the hands and was clinically diagnosed with a Haas-type polysyndactyly. No other abnormalities were recorded. In family 2 father and son presented with complete cup shaped polysyndactyly of the hands. On both hands a total number of six metacarpal bones were diagnosed. The

Table 1. Microduplications of various sizes encompassing the ZRS are associated with Haas-type polysyndactyly and Laurin-Sandrow syndrome

Family	Genomic location (hg19)	Size of duplication (kb)	Phenotype
1	Chr7:156,437,229-156,692,706	~255	PS Haas-type/SD4
2	Chr7: 156,491,887-156,671,016	179	PS Haas-type/SD4, partial syndactyly of toe III–V
3	Chr7: 156,570,780-156,646,750	~75	LSS: total syndactyly of hands, mirror image duplication of feet, nasal defect
4	Chr7: 156,563,856-156,610,632	~47	LSS: total syndactyly of hands, mirror image duplication of feet
5	Chr7: 156,578,108-156,594,751	16	LSS: total syndactyly of hands, mirror image duplication of feet, nasal defect

PS, polysyndactyly, SD4, syndactyly type IV, LSS, Laurin-Sandrow syndrome.

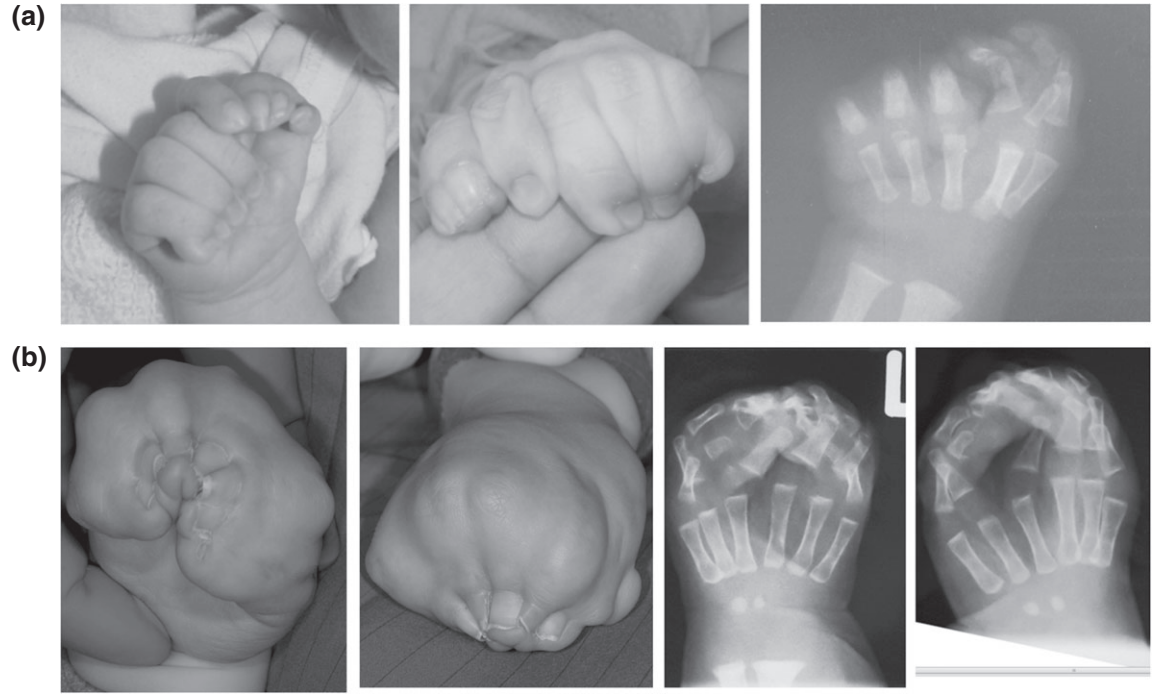

Fig. 1. Families 1 and 2 with clinical signs of Haas-type polysyndactyly. (**a**) Family 1 is a non-consanguineous family from Peru. The affected boy showed a polysyndactyly of the both hands including pre-axial and post-axial polydactyly and was clinically diagnosed with a Haas-type polysyndactyly. No other abnormalities were recorded. The radiograph shows the left hand of the affected boy. (**b**) In family 2 father and son presented with complete cup shaped polysyndactyly of the hands including bilateral hexadactyly. The feet showed partial syndactyly of toes III–IV and complete syndactyly of IV-V. Pictures and radiographs show hands of the affected son.

fusions of the proximal to distal phalanges were in both patients completely osseous. The feet showed a comparably mild phenotype with partial syndactyly of III/IV and complete syndactyly of IV/V.

Families 3–5 showed the characteristic features of LSS including complete polysyndactyly of the hands, mirror image polysyndactyly of the feet, and duplication of the fibula together with absence of the tibia (Fig. 2). Family 3 with one affected boy originates from India. He presented with bilateral polysyndactyly of the hands and mirror image polysyndactyly of the feet. Radiograph of the feet showed symmetrical mirror image polydactyly of the metatarsal bones and toes, with the right foot showing 12 toes and the left foot showing 10 toes. The pelvic bones and femur were normal but the fibula was duplicated and the tibia was absent. The nose showed underdeveloped alae nasi, broad tip, and short grooved columella (11).

Family 4 is a consanguineous family from Pakistan with three affected individuals (father and two of his daughters). All affected showed cup-shaped polysyndactyly of the hands and mirror image polydactyly of the feet. The syndactylies of the hands were of osseous and cutaneous nature, one could recognize based on the existing images. A radiological examination of the lower limb was not available.

Family 5 (affected father and son) has previously been described (10). Both presented with hypoplastic alae nasi, bilateral complete syndactyly of the hands, and preaxial mirror image polysyndactyly of the feet.

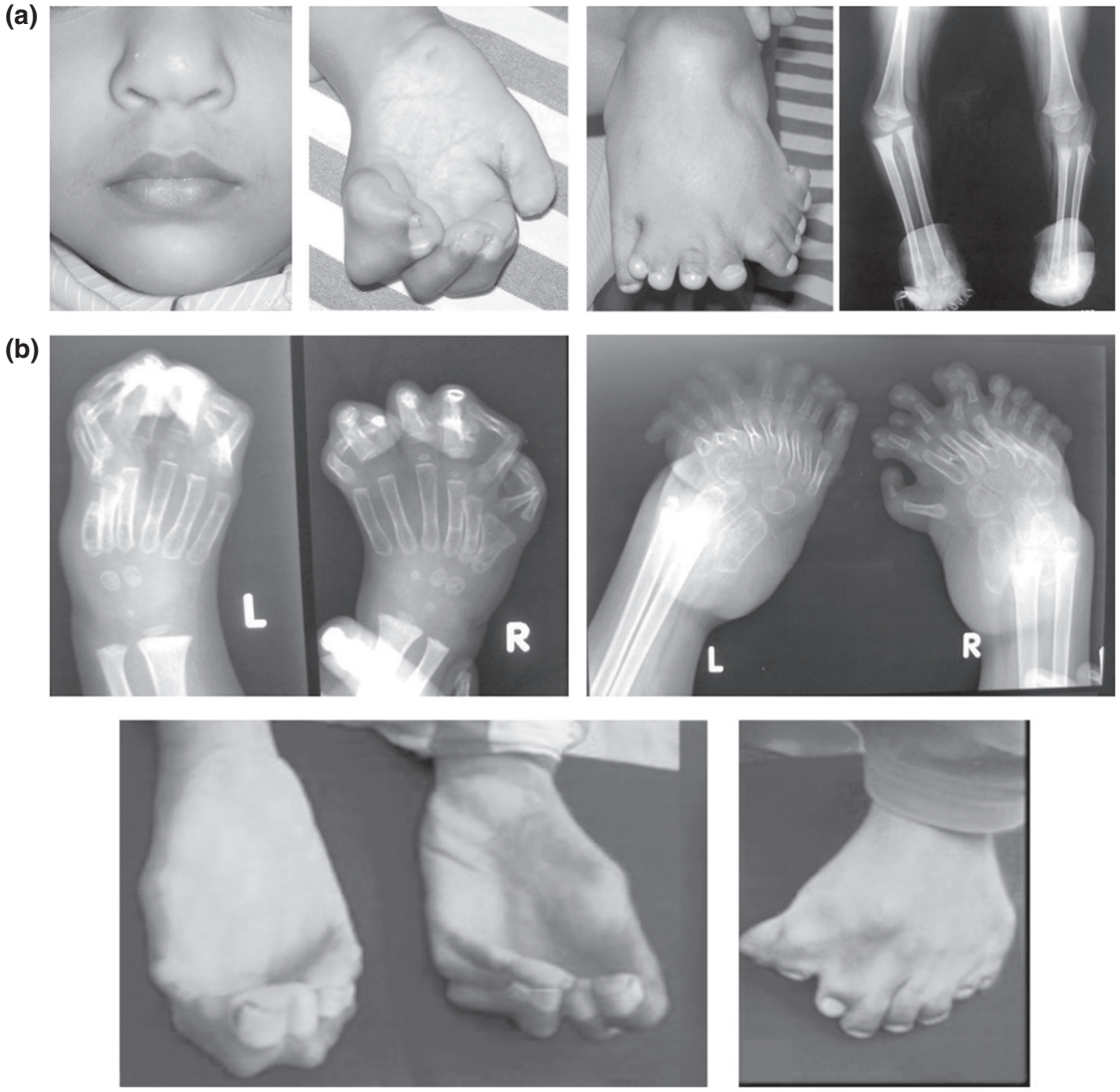

Fig. 2. Families 3 and 4 show the characteristic features of Laurin-Sandrow syndrome (LSS) including complete syndactyly of the hands and mirror image polysyndactyly of the feet. (**a**) Family 3 with one affected boy who presented with nasal defects, bilateral polysyndactyly of the hands and mirror image polysyndactyly of the feet. Radiograph of the feet showed symmetrical mirror image polydactyly of the metatarsal bones and toes as well as duplication of the fibula. (**b**) Family 4 is a consanguineous family from Pakistan with three affected individuals. All affected showed a cup-shaped polysyndactyly of the hands combined with mirror image polysyndactyly of the feet.

The thumb appeared hypoplastic, all fingers were fused (osseus fusion of the distal phalanges) and the second phalanges were rounded and differ in size. The son shows additional flexion contractures of hand wrists. Radiographs of the feet showed a broadened talus, duplicated calcanei, nine metatarsals and toes. The pelvic bones and femur were normal but the fibula was duplicated and no tibia was present.

Apart from the congenital hand malformations no abnormalities of the upper limbs were observed in the affected individuals. Psychomotor development was also normal in all patients.

All individuals studied gave informed consent. The study was approved by the Charité Universitätsmedizin Berlin ethics committee.

Microarray-based comparative genomic hybridization (array CGH)

All experiments were carried out with genomic DNA extracted from blood samples. Array CGH was carried out using a whole genome 244 K or 1 M oligonucleotide array (Agilent, Santa Clara, CA) and a custom-designed oligonucleotide array (Roche NimbleGen, Inc., Madison, WI) which covers the critical region from 219.5 to 222.2 Mb on human chromosome 7q36 at high density (average probe spacing 8 bp) (positions according to hg18). 244 K and 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). Analysis settings: aberration algorithm: ADM-2; threshold: 6.0; window size: 0.2 Mb; filter: five probes, log2ratio = 0.29. Custom arrays were analyzed

using the NIMBLESCAN software and circular binary segmentation algorithm for aberration detection. The genomic profile was visualized by the SIGNALMAP software (SIGNALMAP v1.9.0.03, NimbleGen Systems Inc., Madison, WI). Data were submitted to the DECIPHER database (http://decipher.sanger.ac.uk); accession numbers: IWZ281649, IWZ281652, IWZ281654, IWZ281655, and IWZ281656.

Quantitative real-time PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed as described previously (12) using genomic DNA of the index patients and further family members to confirm the duplication detected by array CGH and to show segregation with the phenotype. Primer sequences are given in Table S1, Supporting Information.

Breakpoint analysis

The exact determination of the duplication size and orientation was performed by breakpoint spanning PCR following the qPCR analyses and sequencing of the junction fragment (primer sequences and positions in Table S1).

PCR was performed in a total volume of 20 µl with 40 ng genomic DNA as template, 2 µl 10× buffer, 0.6 µl deoxyribonucleotide (dNTP) mix (10 mM), 0.5 µl primer (10 pMol/µl), 0.6 µl $MgCl_2$ (50 mM), 0.2 µl Taq polymerase (Rapidozym, Berlin, Germany). PCR conditions are available upon request. The PCR products were purified by enzymatic treatment (Exonuclease I, NEB, Ipswich, MA; Shrimp Alkaline Phosphatase, Roche Diagnostics, Mannheim, Germany). For the sequencing of the PCR products the BigDye v3.1 (Applied Biosystems, Foster City, CA) sequencing kit was used. PCR products were analyzed by capillary automat ABI3730 (Applied Biosystems). The sequencing results were processed by DNA-STAR software (DNA-STAR, Madison, WI).

Results

Phenotypic analysis

We investigated a total of nine affected from five unrelated families. All showed a similar hand phenotype consisting of severe polysyndactyly which resulted in cup-shaped hands and in some cases fusion of the nails (synonychia). The feet, however, showed different phenotypes: only minor abnormalities in some consisting of partial syndactyly, whereas the other group had severe mirror image polydactyly of the feet with 10 and more toes on each foot. In addition, in this latter group other bones of the feet such as the calcaneus were duplicated. In two individuals radiographs of the lower legs showed two bones of similar appearance which was interpreted as the absence of the tibia and a duplication of the fibula. The first group was diagnosed with Haas-type polysyndactyly, whereas the more severe phenotype with the mirror image feet was consistent with the diagnosis of Laurin-Sandrow syndrome.

Microduplications in 7q36 are associated with Haas-type polysyndactyly and LSS

Five unrelated investigated families show clinical features that overlap with the phenotypes described as ZRS-associated syndromes, leading to the assumption of a genetic change affecting the ZRS region (chromosome 7q36). A screening for copy number variants (CNVs) within the 7q36 chromosomal region with array CGH detected microduplications of the ZRS region in intron 5 of *LMBR1* on chromosome 7q36 upstream of *SHH* in all affected individuals (Table 1, Fig. 3). Families 1 and 2 affected with Haas-type polysyndactyly showed duplications of ~255 and 179 kb, respectively. Those families with polysyndactyly and mirror image duplications of the feet and duplicated fibula, i.e. Laurin-Sandrow syndrome, had smaller duplications: ~75 kb (family 3), ~47 kb (family 4), and 16 kb (family 5) (Fig. 3).

The array CGH results were confirmed by qPCR in the index patients. Analysis of further affected and unaffected family members of all five families showed that the microduplications segregate with the phenotype i.e. unaffected family members had a normal copy number of the region (Fig. S1). The qPCR data showed one additional copy of the analyzed amplicons i.e. a duplication [relative quantification (RQ) ≥ 1.5] in all affected individuals. Similar duplications were not observed in control DNA samples without limb phenotypes.

All five duplications encompass exon 5 of *LMBR1* and the ZRS element. The larger aberration in family 1 contains in addition to the complete *LMBR1* several exons of *RNF32* and in family 2 as well as family 3 the duplications also include several exons of *LMBR1*. The distance between *SHH* and the duplications ranges from 832 to 973 kb (Fig. 3). These results suggest that Haas-type polysyndactyly is associated with larger duplications of the ZRS region, whereas the LSS phenotype is caused by smaller duplications (<80 kb).

Breakpoint analysis identifies direct tandem duplication

We determined the exact breakpoints and orientation of the microduplications by amplification of a junction fragment that included the transition site between the telomeric and centromeric breakpoints and subsequent sequencing in affected individuals. Junction fragments were detectable only in the affected, but not in the unaffected individuals. For families 2 and 5 the exact breakpoint was identified. For family 2 the duplication ranges from 156,184,648 to 156,363,777 nt (~179 kb), the duplication in family 5 ranges from 156,270,869 to 156,287,512 nt (~16 kb). In both cases we detected microhomology at the breakpoints; three nucleotides (GTA; family 2) and two nucleotides (CA; family 5), respectively, (Fig. S1). Both aberrations are oriented in direct tandem.

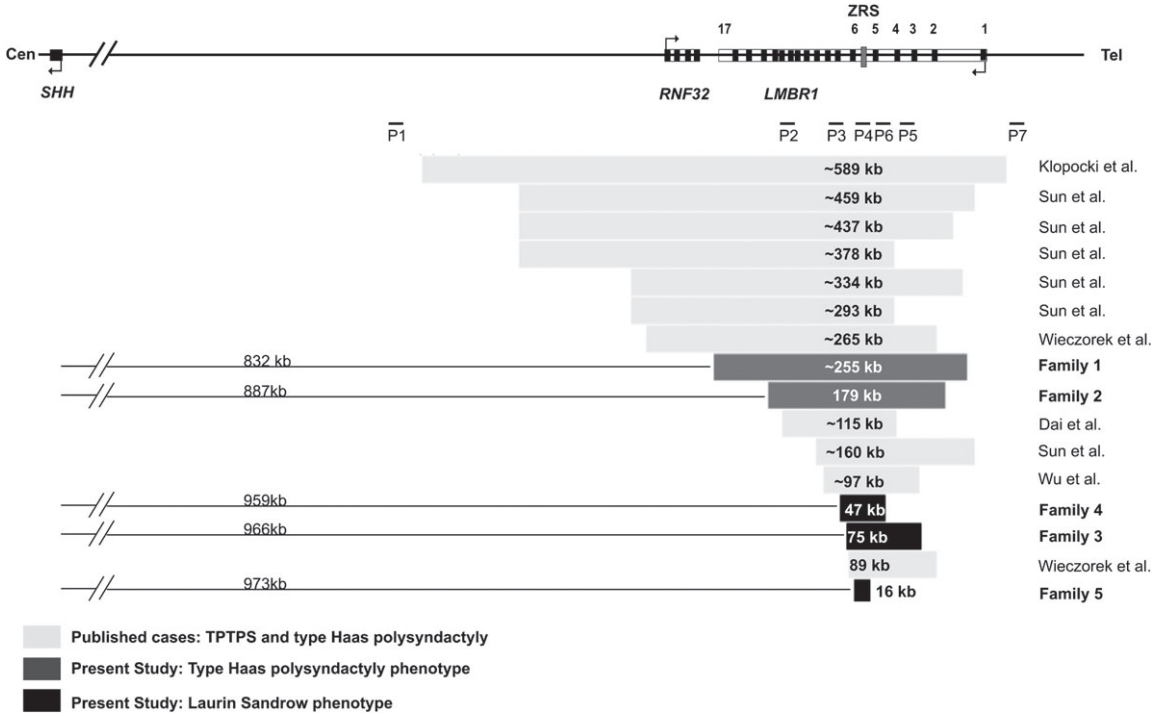

Fig. 3. Schematic representation of the microduplications on 7q36. So far only 11 families have been described with duplications of the ZRS. We show that duplications of the ZRS region >80 kb seem to be associated with Haas-type polysyndactyly, whereas smaller duplications of the ZRS (16–75 kb) result in the Laurin-Sandrow syndrome (LSS) phenotype including mirror image polysyndactyly of the feet and duplication of the fibular.

Discussion

Here we report five unrelated families with overlapping microduplications of the *SHH* limb enhancer ZRS on chromosome 7q36. Clinically, the polysyndactyly phenotype of the hands showed similarities to Apert syndrome and Haas-type polysyndactyly. The latter has been described in association with mutations in the ZRS and duplications of the ZRS in four other families (6, 13). Mutations and duplications involving the ZRS have also been described in triphalangeal-thumb-polysyndactyly syndrome (TPTPS), a condition characterized by preaxial polydactyly with/without triphalangeal thumb and syndactyly of other fingers, and Werner mesomelic dysplasia which features hand phenotypes similar to TPTPS with thumb anomalies and syndactyly as well as hypoplasia of the tibia and foot anomalies. Thus, duplications (so far 11 reported families) as well as mutations of the ZRS can be associated with a range of limb phenotypes (6, 14). The previously published maximum duplication sizes range from ∼589 (4) to ∼89 kb (5) (Fig. 3). The largest duplication has been reported in association with TPTPS and the smallest with Haas-type polysyndactyly. No phenotype/genotype correlation was discernible.

Compared with the previously described cases family 5 of our patient cohort with the LSS phenotype has the up to now smallest duplication (16 kb), but shows the most complex phenotype, with a complete syndactyly of the hands, an aplasia of the patella, a loss of identity between the long bones (tibia and fibula) and a mirror image polydactyly of the feet. Klopocki et al. (2008) have described the largest known duplication on 7q36.3 (589 kb), but the phenotype in this case is TPTPS. In contrast to previous results, our data indicate a correlation between duplication size and phenotype. Apparently, very small duplications are associated with a more severe phenotype. We expand the clinical phenotype of the ZRS-associated syndromes and propose that larger duplications of the ZRS containing region (89–589 kb) are associated with Haas-type polysyndactyly, Werner mesomelic dysplasia, or TPTPS, whereas smaller duplications of the ZRS (16–75 kb; <80 kb) result in LSS. Furthermore, our clinical data show that LSS is a well-defined condition characterized by severe cup-shaped polysyndactyly of the hands, mirror image polydactyly of the feet and aplasia of the tibia with duplication of the fibula (Fig. 3).

The lower limb phenotype in LSS is overlapping with the clinical phenotype associated with *PITX1* deletions i.e. mirror image polydactyly of the feet (15) which hints at a functional link between *SHH* expression and *PITX1*. However, data from *Pitx1* −/− mice show no effect on *Shh* expression in the limb bud (16) and in contrast to the LSS cases patients with *PITX1* deletions do not show any hand phenotypes.

The mechanism how the ZRS influences the expression pattern of *SHH* has been subject of intense investigation. Recently, it was shown that members of two groups of E26 transformation-specific (ETS) transcription factors (ETV4/ETV5) act directly at the ZRS mediating a differential effect on *Shh*, defining its spatial

expression pattern (3). Mutation experiments in transgenic mouse assays revealed that changes in the balance between activating and repressing transcription factor binding sites in the ZRS affected the relative balance of *Shh* expression in the anterior and posterior margins of the limb. Point mutations within ZRS can disturb this delicate balance and result in ectopic expression of *SHH* in the anterior margin of the limb bud. This results in a 'double dose' of hedgehog signaling from the posterior as well as the anterior side of the limb bud which induces polydactyly.

The mutation mechanism of duplications of the ZRS is less clear. Duplications involving regulatory elements have been described for a number of loci (17). According to more recent data there does not seem to be a common mechanisms for this type of CNVs. For example, duplications involving regulatory elements of the *IHH*, *SOX9* and *BMP2* loci are, based on the phenotype, likely to result in misexpression and/or overexpression (18–20). However, duplications may also alter the regulatory architecture of the region by placing enhancers in front of other genes thereby producing misexpression, or by altering the boundaries of regulatory domains. Furthermore, it is possible that the different sizes of duplications affect the efficiency with which the ZRS region interacts with *Shh*. The sizes of aberrations could affect the loop formation of DNA in a different way and thereby influence the *Shh* expression negatively. Mechanisms that have to be considered in this respect have recently been reviewed by us (21). The similarity of phenotypes resulting from ZRS point mutations and duplications suggests similar pathogenic mechanisms. Thus, the duplications are probably to result in ectopic expression of *SHH*. However, how such misexpression could be induced remains unclear.

Lettice et al. recently introduced the term 'enhancer adoption' for long-range *cis*-regulatory mutations, in which ectopic expression of a gene is driven by an enhancer that is not its own because of structural variations (22). We were able to confirm that this mutation mechanism can result in severe misexpression of a gene i.e. *PITX1* causing a congenital disorder (23). Therefore, one could speculate that tandem duplications of the ZRS duplicate not only the ZRS but also other not yet identified *cis*-regulatory element nearby thereby altering the *SHH* regulatory landscape. Another hint for the existence of additional limb specific *SHH cis*-regulatory elements comes from the genetic analysis of a condition called acheiropodia (MIM 200500), a rare, recessive condition in which hands and feet are completely missing. Even though the phenotype is very similar to that seen in mice lacking ZRS (24), the acheiropodia patients carry a homozygous deletion of exon 4 of the *LMBR1* gene which does not involve the ZRS and is located more than 30 kb to the telomeric side.

This report shows that a spectrum of limb malformation syndromes i.e. Haas-type polysyndactyly/syndactyly type 4 (SD4), TPTPS and the Laurin-Sandrow syndrome are associated with genomic aberrations encompassing the ZRS. We postulate in accordance with previous results that these aberrations affect the spatial expression pattern as well as dosage of *SHH* during limb bud development. As a consequence stochastic effects and misregulation of other downstream targets and signaling pathways most probably contribute to phenotypic variability in these syndromes. Wieczorek et al. (2010) introduced a classification of ZRS-associated syndromes: type I (point mutations within ZRS), type II (microduplications of ZRS). However, currently type II includes only complex polysyndactyly with triphalangeal thumb. We suggest to extend the classification for type II ZRS-associated syndromes and differentiate between duplications >80 kb (Haas-type polysyndactyly and TPTPS) as type IIa and duplications <80 kb associated with Laurin-Sandrow syndrome as type IIb. We expand the clinical phenotype of the ZRS-associated syndrome type II and introduce an extended classification, i.e. type IIb LSS phenotype.

Supporting Information

The following Supporting information is available for this article:

Fig S1. Confirmation of microduplications on 7q36 by qPCR. An RQ 1.0 indicates normal copy number i.e. two copies. An RQ value of 1.5 displays three copies of an analyzed amplicon. The analyzed families are illustrated by different colored bars. For families 2 and 5 a breakpoint analysis could be made. Both families show microhomology at the breakpoints on 7q36 marked by boxes [family 2 three nucleotides (GTA), family 5 two nucleotides (CA)]. Telomeric and centromeric reference sequences are indicated on top (tel. ref. seq. and cen. ref. seq.).
Table S1. Quantitative real-time polymerase chain reaction (qPCR) primers (**a**) and sequencing primers for breakpoint detection in families 2 and 5 (**b**).

Additional Supporting information may be found in the online version of this article.

Acknowledgements

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