ORIGINAL ARTICLE

Microdeletions on 6p22.3 are associated with mesomelic dysplasia Savarirayan type

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ABSTRACT

Introduction Mesomelic dysplasias are a group of skeletal disorders characterised by shortness of the middle limb segments (mesomelia). They are divided into 11 different categories. Among those without known molecular basis is mesomelic dysplasia Savarirayan type, characterised by severe shortness of the middle segment of the lower limb.

Objective To identify the molecular cause of mesomelic dysplasia Savarirayan type.

Methods and results We performed array comparative genomic hybridisation in three unrelated patients with mesomelic dysplasia Savarirayan type and identified 2 Mb overlapping de novo microdeletions on chromosome 6p22.3. The deletions encompass four known genes: *MBOAT1*, *E2F3*, *CDKAL1* and *SOX4*. All patients showed mesomelia of the lower limbs with hypoplastic tibiae and fibulae. We identified a fourth patient with intellectual disability and an overlapping slightly larger do novo deletion also encompassing the flanking gene *ID4*. Given the fact that the fourth patient had no skeletal abnormalities and none of the genes in the deleted interval are known to be associated with abnormalities in skeletal development, other mutational mechanisms than loss of function of the deleted genes have to be considered. Analysis of the genomic region showed that the deletion removes two regulatory boundaries and brings several potential limb enhancers into close proximity of *ID4*. Thus, the deletion could result in the aberrant activation and misexpression of *ID4* in the limb bud, thereby causing the mesomelic dysplasia.

Conclusions Our data indicate that the distinct deletion 6p22.3 is associated with mesomelic dysplasia Savarirayan type featuring hypoplastic, triangular-shaped tibiae and abnormally shaped or hypoplastic fibulae.

INTRODUCTION

Mesomelic dysplasias are a group of skeletal disorders characterised by shortness of the middle limb segments (mesomelia). Currently, 11 different mesomelic and rhizo-mesomelic dysplasias are listed in the Nosology and Classification of Genetic Skeletal Disorders.[1] So far the underlying molecular defect has been identified in seven mesomelic and rhizo-mesomelic dysplasias. Among those without known molecular basis are mesomelic dysplasia Nievergelt type (Nievergelt syndrome, MIM 163400) and mesomelic dysplasia Savarirayan type (MIM 605274). Steichen-Gersdorf *et al*[2] described an infant with a phenotype similar to Nievergelt mesomelic dysplasia carrying a 500 kb microdeletion on chromosome 2q11.1 containing the gene *LAF4*. The patient additionally suffered from severe central nervous system dysfunction, and complex malformations of the urogenital tract, resulting in death at the age of 4 months.[2] A more detailed analysis of the deleted region demonstrated that several exons of the *LAF4* gene were deleted in this case, resulting in a truncated LAF4 protein.[3]

Nievergelt syndrome is characterised by severe mesomelic shortness and a triangular or rhomboid shape of the tibiae accompanied by synostosis of the tarsal and metatarsal bones, clubfeet and dysplastic ankle joints.[4] Short radii, restricted mobility of the elbows, radio-ulnar synostosis and dislocated radial heads are also present. Patients with mesomelic dysplasia Savarirayan type share radiographic features with Nievergelt syndrome such as triangular tibiae and dislocated radial heads. The distinct features however are absence of relative fibular overgrowth and tarsal/metatarsal synostoses as well as presence of other skeletal changes including an abnormal pelvis with dislocated hips.[5 6]

In this study, we show that the distinct deletion 6p22.3 is associated with mesomelic dysplasia Savarirayan type featuring hypoplastic, oval-shaped or triangular-shaped tibiae and abnormally shaped, hypoplastic or absent fibulae.

CLINICAL REPORTS

Patient 1

Detailed clinical and radiological findings of this patient were published by Yasui *et al*[7] (case 2). A 1-year-old boy presented with severe mesomelic shortness of the legs. The parents were non-consanguineous and there were no teratogenic exposures during the pregnancy. The family history was unremarkable. Born at 38 weeks of gestation, his birth weight (2.456 g, −2.1 SD) and length (39.6 cm, −3.7 SD) were abnormal. His motor and speech development was normal. Physical examination at the age of 9 months showed multidirectional instability of knees and ankles, without apparent foot abnormalities. Radiographs of the legs revealed bilateral absence of tibia and fibula. Femora were well developed. There was congenital dislocation of the hips. Tarsal bones were developed normal.[7] Radiographs, taken at the age of

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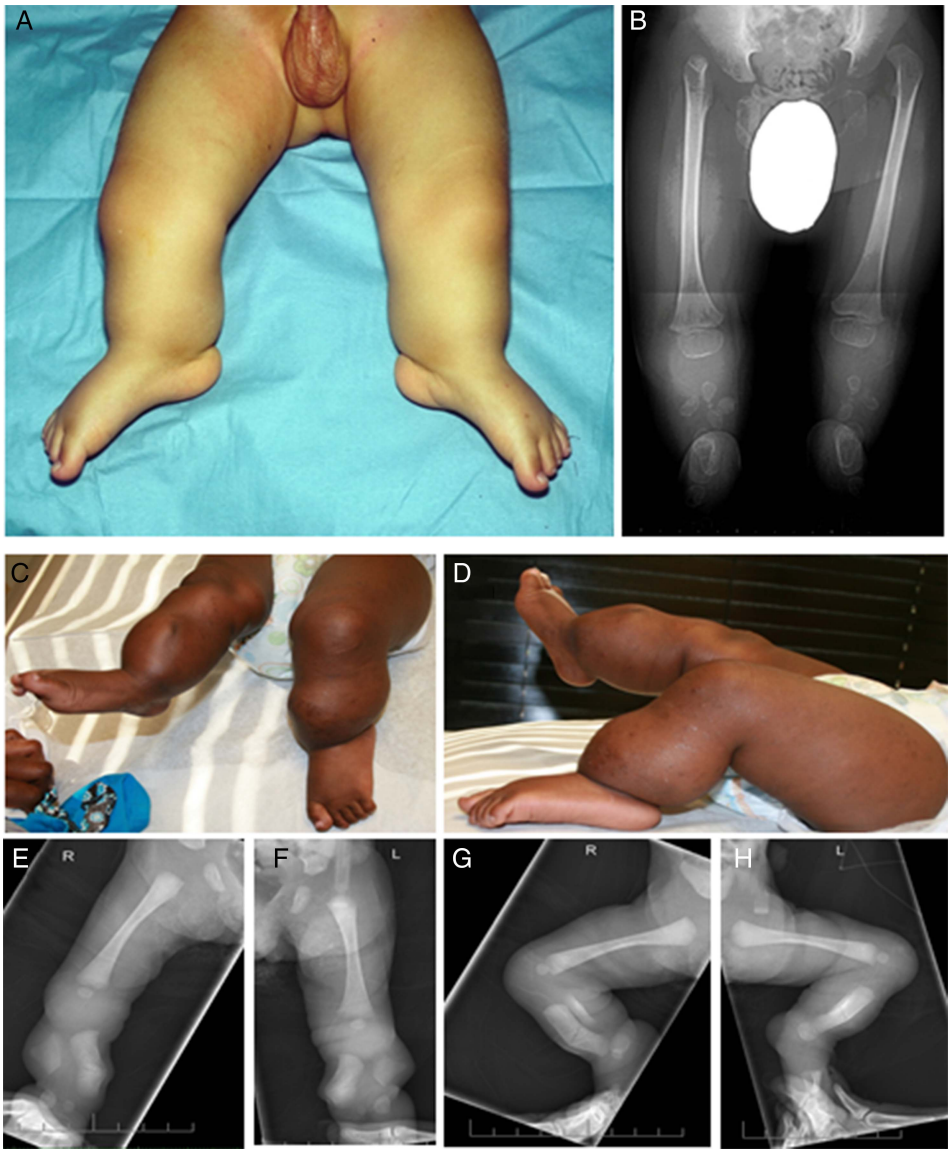

Figure 1 Images of patients. Patient 1: note severe mesomelic shortness of the legs (A). Radiograph at the age of 9 months shows hip dislocation, rounded distal femoral epiphyses and two ossifications centres of tibia and fibula (B). Patient 3: note severe mesomelic shortness and bowing of the lower legs (C and D). Radiographs showing markedly short, and dysplastic tibiae and fibulae with triangular shape in ap view as well as hypoplastic pubic bones. The fibulae were more severely affected than the tibiae (E–H).

2 years and 10 months, showed two uniting ossifications centres of tibia and fibula (figure 1A, B).

Patient 2

This patient was published clinically as 'case 1' by Savarirayan *et al* in 2000.[5] The girl was the third child of healthy non-consanguineous parents, and family history was unremarkable. Severe shortness of the lower legs was noted at birth. Development was delayed with crawling at 15 months and first spoken words at the age of 21 months and she required special education. Prominent radial heads and short broad forearms were observed. Supination and pronation of forearms was incomplete. Bilateral equinovalgus foot deformities were present.

Radiographic findings included proportionate shortness of radii and ulnae, absence of fibulae and marked hypoplasia of the tibiae, which were triangular or oval in configuration with the apices/bases projecting towards the skin.

Patient 3

Patient 3 presented at 11 months old with severe mesomelic shortness of the lower legs. Her lower limb anomalies were first identified on prenatal ultrasound at 5 months' gestation. There were no teratogenic exposures during the pregnancy. She was the only child of non-consanguineous parents. Her father, paternal grandfather and a paternal cousin had bowed legs, but there were no family members with a history of significant limb anomalies. Her development was normal except for gross motor delays related to her lower limb anomalies. Physical examination revealed a normal appearance to her arms. Her lower legs revealed significant mesomelic shortness with anterior tibial bowing. Radiographic findings included hypoplasia of the tibiae and the fibulae, which were very short with a nearly triangular shape at the ap view. The apices projected towards the skin and the fibulae were more severely affected (figure 1C–H). Pubic bones were hypoplastic. Radius and ulnar were well developed.

Patient 4

This patient presented at age 5 years with mildly delayed motor and expressive speech developmental delay. Growth parameters are along 90–97th centile with normal limb proportions. She had pedes plani but no other skeletal abnormalities. Therefore, no radiographs were obtained.

METHODS

Human material

Venous blood samples were obtained from the patients by standard procedures.

Microarray-based comparative genomic hybridisation (array CGH)

All experiments were done with genomic DNA extracted from blood samples.

Array comparative genomic hybridisation (array CGH) for patient 1 was carried out using a submegabase whole human genome tiling path BAC array as previously described.[8] Images were scanned using GenePix 4100A and analysed using GenePix Pro V.6.0 software (Axon Instruments, Foster City, California, USA). Further analyses and visualisation were performed with CGHPRO.[9] Also, 35 882 BACs were included in the analysis. Raw data were normalised by 'Subgrid LOWESS'. The log 2 ratio of test to reference was calculated and plotted according to chromosomal position of the clones. Copy number gains and losses were determined by using a conservative threshold of 0.3 and −0.3, respectively. Aberrant signals including three or more neighbouring BAC clones were considered as genomic aberrations.

The array CGH result was confirmed by FISH using BAC probes (RP11-86O17, RP11-204E9) located within the deletion on chromosome 6p. The interphase FISH analysis showed only one signal for the probe RP11-86O17 in 274/339 interphase nuclei (81%) and only one signal for the probe RP11-204E9 in 88/102 interphase nuclei (86%), respectively. The testing of parents revealed two signals for both probes on chromosome 6 in both parents.

Array CGH for patient 2 was carried out using a whole-genome 1 M oligonucleotide array (Agilent, Santa Clara, California, USA).Also, 1 M arrays were analysed by Feature Extraction V.9.5.3.1 and CytoGenomics V.2.7.8.0 (Agilent, Santa Clara, California, USA). Analysis settings—aberration algorithm: ADM-2; threshold: 6.0; window size: 0.2 Mb; filter: 5probes, log 2 ratio=0.29. Array CGH for patient 3 was carried out using a whole-genome human SNP Array V.6.0 by Affymetrix, and for patient 4 and the parents a whole genome 180k CytoSure ISCA array was used. Data were submitted to the DECIPHER database (http://decipher.sanger.ac.uk); accession numbers: 304175, 304177, 304179 and 288102.[10]

Quantitative real-time PCR (qPCR)

qPCR was performed as described previously[11] using genomic DNA of the index patients and their parents to confirm de novo status. Primer sequences are available upon request.

Sanger sequencing analysis

Genomic DNA from the patients was extracted from peripheral blood leucocytes using standard protocols. Coding region and flanking intronic sequences of the exons of *MBOAT1*, *E2F3*, *CDKAL1* and *SOX4* were amplified and sequenced. 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 deoxynucleotide mix (10 mM), 0.5 µL primer (10 pMol/µL), 0.6 µL $MgCl_2$ (50 mM) and 0.2 µL Taq polymerase (Rapidozym, Germany). Primers and 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 V.3.1 (Applied Biosystems) sequencing kit was used. PCR products were analysed by capillary automat ABI3730 (Applied Biosystems). The sequencing results were processed by DNA-STAR software (DNA-Star).

Databases and in silico analysis

We used the databases DECIPHER (https://decipher.sanger.ac.uk/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and DGV (http://dgv.tcag.ca/dgv/app/home) to classify the deletions.[10 12 13]

The processing of the Hi-C data was performed by the Ren lab[14] and downloaded via http://chromosome.sdsc.edu/mouse/hi-c/download.html.

The Gene Expression Omnibus accession numbers for the ChIP-sequencing data for the H3k27ac enhancer mark reported by Cotney *et al*[15] are GSE42413 and GSE42237.

RESULTS

In this study, we performed array CGH in three unrelated patients with mesomelic dysplasia Savarirayan type and identified 2 Mb overlapping de novo microdeletions on chromosome 6p22.3. In a fourth patient with intellectual disability but without skeletal abnormalities, we detected an overlapping slightly larger do novo deletion also encompassing the flanking gene *ID4*.

For patient 1, array CGH revealed a deletion on chromosome 6 [arr[hg19] 6p22.3 (19964281–22008341)x1] (figure 2). The size of the deletion was about 2 Mb encompassing four known genes: *MBOAT1*, *E2F3*, *CDKAL1* and *SOX4*. The result was confirmed by FISH using BAC probes located within the deletion on chromosome 6. Testing of parents revealed two signals for both probes on chromosome 6 in both parents (see online supplementary figure S1). Therefore, the deletion occurred de novo in patient 1. For patients 2 and 3, array CGH revealed a similar 2 Mb deletion as in patient 1 [arr[hg19] 6p22.3 (19974194–22013061)x1] and [arr[hg19] 6p22.3 (19849280–21604600)x1], respectively (figure 2). The results were confirmed by qPCR. In patient 2, the parents were not available for testing, whereas qPCR analysis of the parents revealed a de novo status in patient 3 (see online supplementary figure S1).

For patient 4, array CGH revealed a 2.5 Mb de novo deletion on chromosome 6 [arr[hg19] 6p22.3 (19153 386–21698497) x1] (figure 2). The result was confirmed by qPCR, and the deletion encompassed the five genes: *MBOAT1*, *E2F3*, *CDKAL1*, *SOX4* and *ID4*. qPCR analysis of the parents revealed a de novo status (see online supplementary figure S1). The patient also harboured a 540 kb duplication on 8p23.1 of unknown significance. This patient did not show any skeletal abnormalities. All positions are listed in detail table 2.

The overlapping deletions on chromosome 6p22.3, as well as another case published by Landinsky *et al*[16] in 2014, are shown in figure 3. Mutations on the second allele were excluded by sequencing of all exons and splice sites of patients 2 and 3. The phenotypic characteristics of our patients and the patient described by Ladinsky *et al* are compared in table 1.

The DECIPHER database lists four other patients with overlapping, but much larger, deletions.[10] None of those patients had significant skeletal abnormalities. In two of them, the phenotype was given in detail and did not include any significant skeletal abnormalities (accession numbers: 249613 and 263354). The deletions were much larger with 4.8 and 10.6 Mb and encompassed 20 and 68 genes, respectively. One patient was diagnosed with intellectual disability, autism, downslanting palpebral fissures and muscular hypotonia, whereas the other was described to have several dysmorphic features and atrophy/degeneration of corpus callosum. Inheritance and pathogenicity

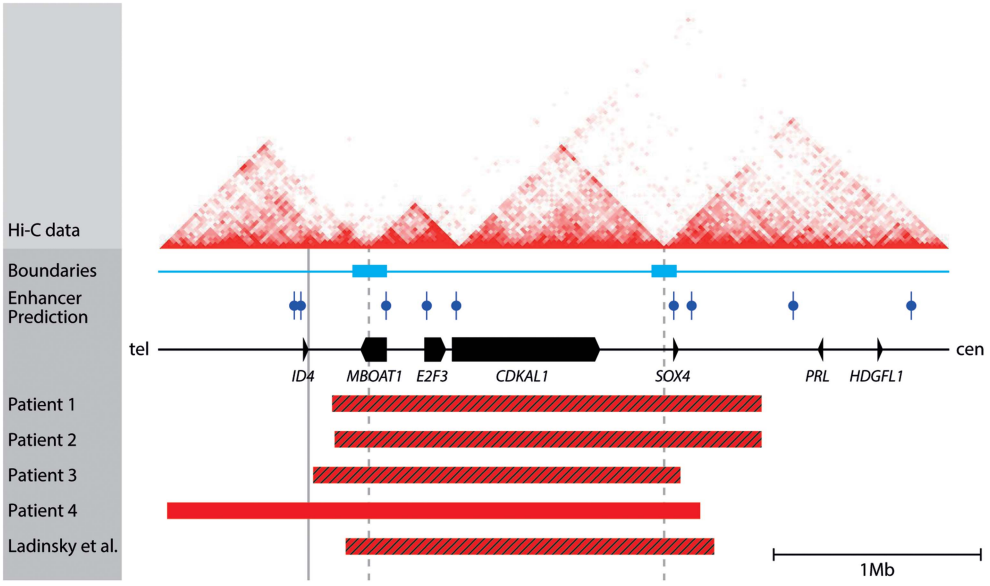

Figure 2 Genomic locus of the deleted region 6p22.3. Centromeric is right, telomeric left. Genes and their direction of transcription are indicated by black boxes/arrows. Potential boundaries of topologically associated domains (TADs) (red triangles, top) according to Dixon *et al*[14] are indicated by light blue boxes on top. Thin broken vertical lines indicate the TAD boundaries. H3k27ac enhancer marks (enhancer prediction) are indicated by blue marks above the gene symbol.[15] Note that several of these marks are located centromeric of the deletion. The deletions reported here are shown below the gene symbols, indicated by red/black bars (patients 1, 2 and 3). A grey vertical line indicates that ID4 is not deleted in patient 3. All three patients showed severe mesomelic shortness of the lower legs. The deletions encompass four protein coding genes: *MBOAT1*, *E2F3*, *CDKAL1* and *SOX4* on chromosome 6p22.3. A similar, recently published family is shown below.[16] In this study, a fourth patient was found to have a slightly larger deletion extending further distal, encompassing also the telomeric gene *ID4*, without any skeletal abnormalities (red bar). The deletions associated with mesomelic dysplasia remove two regulatory boundaries and bring several limb enhancers into close proximity of *ID4*. Thus, the deletions could result in the aberrant activation and misexpression of *ID4* in the limb bud, thereby causing the mesomelic dysplasia.

was listed as unknown and as de novo with unknown pathogenicity, respectively. Two more had no phenotype given, but the responsible clinicians were contacted and it was confirmed that there were no major skeletal abnormalities (accession numbers: 256653 and 285668). Therefore, no radiographs of the legs were obtained. One of these patients was diagnosed with developmental delay, heart defects, short neck and eye abnormalities and was published by Bremer *et al*.[17] The other patient presented with intellectual disability; inheritance was unknown but pathogenicity was listed as definitely pathogenic. The deletions were 7.3 and 4 Mb and encompassed 45 and 20 genes, respectively. ClinVar lists only one overlapping 12 Mb deletion in a patient with developmental delay (dbVar: nsv530892).[12]The database of genomic variants does not list entries that encompass the complete region.[13] The largest described deletion was found in 1 out of 95 healthy individuals and spans 20 kb between *CDKAL1* and *SOX4*. All other annotated structural variations are considerably smaller.

DISCUSSION

We identified three unrelated patients with mesomelic dysplasia and overlapping microdeletions encompassing four protein coding genes: *MBOAT1*, *E2F3*, *CDKAL1* and *SOX4* (figure 3). All patients showed severely hypoplastic tibiae and fibulae. In one case, the fibulae were rhomboid shaped, whereas in the others the fibulae were hypoplastic and fragmented. The radial and ulnar bones were also affected by mild shortness in patient 2. Further signs were hip dislocation and radial head anomalies (radial head dislocation in patient 1 and prominent radial head in patient 2). Considering the many overlapping features, these three cases can be classified as mesomelic dysplasia Savarirayan type. Patient 3 also shares similarities with Nievergelt syndrome in particular with regard to the presence of triangular-shaped fibulae and tibiae. However, in contrast to Nievergelt syndrome, relative elongation of fibulae and tarsal and tibia-fibular synostosis were not present.

Our data show that the distinct deletion at 6p22.3 is associated with mesomelic dysplasia Savarirayan type characterised by hypoplastic, oval-shaped or triangular-shaped tibiae and fibulae. A fourth case with an overlapping microdeletion and striking clinical as well as radiological similarities was recently published.[16] This patient additionally presented with hypereosinophilic syndrome, sensorineural hearing loss due to malformation of the semicircular canals and mild craniofacial dysmorphic features. Our patient 2 was diagnosed with global developmental delay. Patient 4, carrying a de novo 2.5 Mb overlapping deletion encompassing *MBOAT1*, *E2F3*, *CDKAL1*, *SOX4*, and additionally *ID4*, also presented with global developmental delay but without skeletal malformations. Interestingly, the DECIPHER database lists four other patients with overlapping, but much larger, deletions between 4 and 10 Mb.[10] None of those patients had significant skeletal abnormalities, and three were diagnosed with intellectual disability or developmental delay.

The *MBOAT1* (membrane bound O-acyltransferase domain containing 1 gene) gene belongs to the membrane-bound O-acetyltransferase superfamily.[18] It was shown to be disrupted by a translocation in a patient with short stature and brachydactyly.[19] However, no further mutations have been reported in this gene to this date. *E2F3* (E2F transcription factor 3) is expressed in the developing limb buds, but no skeletal abnormalities were reported in knockout mice.[20–22] The protein encoded by *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1) is a member of the methylthiotransferase family. Inactivation in the mouse is associated with abnormalities in insulin secretion but not with abnormal limbs.[23] *SOX4* (sex determining region on Y-box 4) is expressed in the

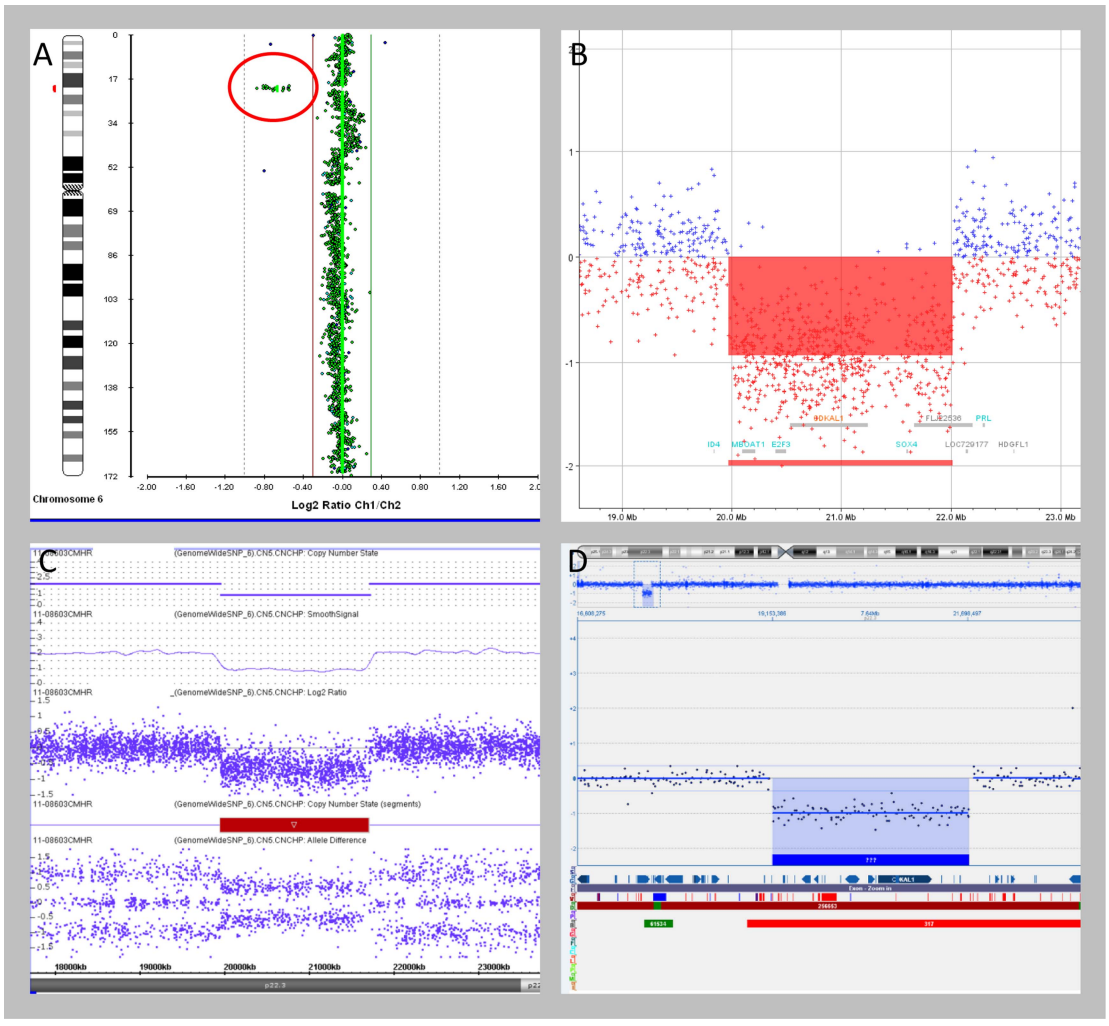

Figure 3 Results of array comparative genomic hybridisation (CGH). Array CGH was carried out using a submegabase whole human genome tiling path BAC array in patient 1: arr[hg19] 6p22.3 (19964281–22008341)x1 (A). Array CGH for patient 2 was carried out using a whole-genome 1 M oligonucleotide array (Agilent, Santa Clara, California, USA): arr[hg19] 6p22.3 (19974194–22013061)x1 (B). Array CGH for patient 3 was carried out using a whole-genome human SNP Array 6.0 by Affymetrix: Patient 3 arr[hg19] 6p22.3 (19849280–21604600)x1 (C). Array CGH for patient 4 was carried out using the whole-genome 180k CytoSure ISCA array: arr[hg19] 6p22.3 (19153386–21698497)x1 (D). All experiments were done with genomic DNA extracted from blood samples. The results and de novo statuses were confirmed by FISH or qPCR (see figure S1) and detailed information for the genomic positions are listed in table 2.

developing limb buds and a key player in cartilage and bone development.[24 25] However, previous studies showed that *SOX4*$^{-/-}$ mice die at embryonic E14.5 from heart defects and do not show any abnormalities of the limbs.[26] The heterozygous knockout mice did not show any obvious phenotypes and were indistinguishably from their wild-type littermates.[20–23 26] None of the patients were reported to have any cardiac abnormalities.

To further classify the genes deleted in our patient, we included the haploinsufficiency score (HI index) of the particular genes[27]: high ranks (eg, 0–10%) indicate a gene is more likely to exhibit HI, and low ranks (eg, 90–100%) indicate a gene is more likely to not exhibitHI.[27] While *MBOAT1* and *CDKAL1* exhibiting scores of 70.2 and 43.9, respectively, are unlikely to cause the phenotype, *E2F3* and *SOX4* are part of bone and limb development and have low HI scores of 4.5 and 2.5. Nevertheless, the absence of limb defects in the *E2F3* and *SOX4* knockout mice makes them unlikely candidates to cause mesomelic dysplasia. Mutations on the second allele were excluded by sequencing of all exons and splice sites.

Patients 2 and 4 showed, in addition to their skeletal abnormalities, developmental delay. All genes within the deletion (MBOAT1, CDKAL1, E2F3 and SOX4) show strong expression during brain development and have to be considered as candidates for developmental delay.[28] The DECIPHER database lists one very small deletion encompassing only one coding exon of CDKAL1 in a patient with neonatal hypotonia and seizures (patient 294851). Neonatal hypotonia could be a sign of early developmental delay and therefore one could speculate that CDKAL1 might be a candidate gene for developmental delay and seizures.

It is striking that patient 4 has no skeletal abnormalities but harbours an overlapping but only slightly larger deletion that also includes the *ID4* gene.

Although it cannot be ruled out that the phenotypic differences between patients 1–3 and patient 4 reflect low penetrance or even variable expressivity of HI of the deleted genes, we think that other molecular mechanisms besides gene-dosage effects have to be considered. One mutational mechanism to be considered is the aberrant activation by enhancers that were moved by the deletion in close proximity to the new target gene, a mechanism known as enhancer adoption.[29] Data from genome-wide chromosome conformation capture analysis indicate that the

Table 1 Phenotypic characteristics of our patients and the patient described by Ladinsky *et al*

	Patient 1	Patient 2	Patient 3	Ladinsky *et al*
Clinical features				
Upper limbs	Bilateral subluxation of radial heads	Short, broad forearms with prominent radial heads and normal hands	Normal	NA
Femur	Normal	Normal	Normal	NA
Patellae	Palpable	Palpable	NA	NA
Lower leg	Mesomelic shortened	Mesomelic shortened with bowing	Mesomelic shortened	Mesomelic shortened
Feet	Normal	Equinovalgus foot deformity	Normal	NA
Radiological findings				
Upper limbs	NA	Proportionate shortness of radii and ulnae; ulnae proximally widened; humeri and phalanges normal	Normal	NA
Hips	Congenital dislocation	Dislocated, ilia increased in height and decreased in width, with widened sacro-sciatic notches; acetabular roofs were hypoplastic	Hypoplastic pubic bones	Left hip dysplasia
Femur	Well-developed, rounded distal femoral epiphysis,	Normal, rounded distal femoral epiphysis	Well-developed	NA
Tibia and fibula	Cartilaginous remnants of tibia and fibula, each with two ossification centres, which united to form short diaphyses (at age 2 years, 10 months)	Absence of fibulae; hypoplastic,oval-shaped tibia abutting the anterior surface of the shin (at age 21 months)	Shortening and deformity of tibiae, hypoplastic and nearly triangular shaped fibulae	Described as 'hemimelia'(apparent hypoplastic and oval-shaped tibiae and absent fibulae)
Feet	Normally developed tarsal bones	Equinus deformities of the feet, tarsal bones and metatarsals were normal	Normal	NA
Other features				
Other features	NA	Bilateral supernumerary nipples, several Mongolian spots	None	Plagiocephaly, facial dysmorphisms, sensorineural hearing loss, heart defect Hypereosinophilic syndrome
Development	Normal	Globally delayed	Normal	NA

Individuals 1, 2 and the patient of Ladinsky *et al* are reported in detail in the original articles.[5 7 16]
NA, information not available.

Table 2 Genomic positions of the deletions on 6p22.3 (arr[hg19])

	Last copy number neutral probe—telomeric	Most distant probes within the deletion on 6p22.3 arr[hg19]	Last copy number neutral probe—centromeric	Deleted genes
Patient 1	19910613	19964281–22008394	22072989	*MBOAT1*, *E2F3*, *CDKAL1* and *SOX4*
Patient 2	19965294	19974194–22013061	22016585	*MBOAT1*, *E2F3*, *CDKAL1* and *SOX4*
Patient 3	19840492	19849280–21604600	21630684	*MBOAT1*, *E2F3*, *CDKAL1* and *SOX4*
Patient 4	19143048	19153386–21698497	21711233	*MBOAT1*, *E2F3*, *CDKAL1*, *SOX4* and additionally *ID4*

Patients 1–3 are affected by mesomelic dysplasia Savarirayan type while patient 4 showed no skeletal abnormalities.

human genome is divided into conserved self-interacting regions called topologically associated domains (TADs), which are separated by regulatory boundary elements.[14] In this situation, the deletion removes the original target gene and allows enhancers from neighbouring domains to ectopically activate nearby genes.[30] A similar pathomechanism was described to cause Liebenberg syndrome, a rare upper limb malformation[31] and autosomal-dominant leukodystrophy.[32] Ibn-Salem *et al*[33] demonstrated that in up to 11% of all deletions reported in the DECIPHER database the phenotype could be best explained by enhancer adoption or a combination of gene-dosage effects and enhancer adoption. In a recent study, Lupiáñez *et al* demonstrated that deletions of TADs boundaries in humans and mice can cause rewiring of long-range regulatory architecture and result in pathogenic phenotypes. Their results provide the molecular framework to understand the impact of enhancer adoption as mutational mechanism in congenital disorders.[34]

To identify potential limb enhancer elements in the region of the deletions, we analysed ChIP-sequencing data published by Cotney *et al*.[15] In their study, they performed genome-wide mapping of the histone modification H3K27ac in human embryonic limb tissue and demonstrated that many of the peaks show in vivo enhancer activity in a transgenic mouse assay.[15] As shown in figure 3, several H3k27ac enhancer marks map into the region of the deletions detected in our patients. We identified at least two potential limb enhancer regions marked by the H3k27ac enhancer mark the map telomeric to the deletions into the regulatory domain of *SOX4* (figure 3). Through the deletion of *SOX4* and two regulatory boundaries, these potential limb enhancer elements are free to act on other neighbouring genes, that is, *ID4*. This may result in misexpression of *ID4* in the limbs causing skeletal abnormalities in the patients. The proposed misexpression is unlikely to be caused by individual enhancer elements but rather by a cluster of enhancer elements (regulatory landscapes) causing enhancer adoption. *ID4* (inhibitor of DNA binding 4) is a member of the inhibitor of DNA binding (ID) protein family and is expressed in the developing limb buds.[20] It was shown to have a molecular function in osteoblast differentiation, and ID4$^{-/-}$ mice showed severe growth retardation and died by 5 weeks.[35] Misexpression of *ID4* in the developing bones may thus have a negative effect on bone growth in the limbs, ultimately resulting in mesomelic dysplasia. However, the exact molecular mechanism of the deletion remains to be elucidated. Deletions of regulatory boundaries and/or cis-regulatory elements could also affect the expression of other genes at the locus such as *PRL*, *HDGFL1*, *NRSN* and *DCDC2* and thereby contribute to the phenotype.

A growing number of non-coding regulatory mutations are being identified in congenital disease.[31–33] Our data in combination with the molecular findings by Lupiáñez *et al*[34] show that mutations and structural variations outside of the coding genome can interfere with normal gene regulation by disrupting the regulatory landscape. Therefore, the regulatory landscape of the genome has also to be taken into consideration when investigating the pathology of human disease. Exome sequencing studies in the remaining mesomelic dysplasias as well as in other skeletal dysplasias have failed to identify coding mutations in many cases. Therefore, regulatory and non-coding mutations need to be considered in the development of these entities. Furthermore, new model systems need to be developed to study the molecular effects of regulatory mutations.

In summary, we show that the distinct deletion 6p22.3 is associated with mesomelic dysplasia Savarirayan type featuring hypoplastic, oval-shaped or triangular-shaped tibiae and abnormally shaped, hypoplastic or absent fibulae.

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Contributors Patient recruitment and phenotyping: RF, CJC, RS, GN, NY, JS, HVE, MJL, DH, SM and MS. Array-CGH experiments and analysis: RF, JW, KK, EK, BRD, AD, SM and MS. All the authors contributed in writing and reviewing the manuscript.

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Microdeletions on 6p22.3 are associated with mesomelic dysplasia Savarirayan type

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