European Journal of Human Genetics (2000) 8, 54–62

www.nature.com/ejhg

ARTICLE

Phenotypic variation and genetic heterogeneity in Léri-Weill syndrome

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Léri-Weill syndrome (LWS) or dyschondrosteosis represents a short stature syndrome characterised by the mesomelic shortening of the forearms and lower legs and by bilateral Madelung deformity of the wrists. Recently, mutations in the pseudoautosomal homeobox gene *SHOX* have been shown to be causative for this disorder. This gene has previously been described as the short stature gene implicated in Turner syndrome (TS). We studied 32 Léri-Weill patients from 18 different German and Dutch families and present clinical, radiological and molecular data. Phenotypic inter- and intrafamilial heterogeneity is a frequent finding in LWS, and phenotypic manifestations are generally more severe in females. In males, muscular hypertrophy is a frequent finding. To test for *SHOX* mutations we used FISH, Southern blot and SSCP analysis as well as long-range PCR and sequencing. We identified (sub)microscopic deletions encompassing the *SHOX* gene region in 10 out of 18 families investigated. Deletion sizes varied between 100 kb and 9 Mb and did not correlate with the severity of the phenotype. We did not detect *SHOX* mutations in almost half (41%) the LWS families studied, which suggests different genetic etiologies. *European Journal of Human Genetics* (2000) **8**, 54–62.

Keywords: Léri-Weill syndrome; Turner syndrome; SHOX; Madelung deformity; short stature

Introduction

Height and weight represent truly classic 'polygenic' characters. Although these traits are most likely determined by different environmental factors and small additive effects of many different genes, major genetic determinants for both height and weight have been characterised in recent years. One of them, *SHOX*, a homeobox-containing transcription factor, has been implicated in the final height of a person[1,2] Haploinsufficiency of *SHOX* has been shown to be causative for the short stature phenotype associated with Turner syndrome and a certain proportion of 'idiopathic' short-stature patients (Rao *et al*[2], and unpublished results, 1998).

Turner syndrome (TS) represents a frequent chromosomal disorder associated with partial or total loss of a sex chromosome. Significant clinical variability exists in the phenotype of females with TS, with gonadal dysgenesis and short stature as the key symptoms of this disorder. Despite the fact that short stature represents one of the leading symptoms, Turner females with normal stature (arbitrarily defined as being above the 3rd percentile of a given population) exist due, for example, to tall parents or other unknown factors. Different somatic features are found at different frequencies in Turner syndrome.[3] Among those, a skeletal deformity of the forearm, termed Madelung deformity, has been seen in up to 8% of females with TS.[4]

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Received 25 May 1999; revised 27 August 1999; accepted 1 September 1999

This abnormality involving the shortening and bowing of the radius with a dorsal subluxation of the distal ulna has also been seen in one other syndrome, Léri-Weill syndrome (LWS). LWS or dyschondrosteosis, formerly thought to be inherited in an autosomal dominant manner, is characterised by mesomelic shortening of the forearms and lower legs and by a Madelung deformity of the wrists.[5] Despite the fact that females are more often and more severely affected than males,[6] two recent studies on 14 families with LWS have implicated the pseudoautosomal gene *SHOX* as causative for this disorder. In total, 12 large-scale deletions encompassing *SHOX* and two *SHOX* nonsense mutations leading to protein truncation were defined.[7,8] Here, we report detailed clinical and molecular genetic data of 32 LWS patients from 18 carefully evaluated families, all with evident Madelung deformity and a remarkable phenotypic variation in other LWS features. Surprisingly, and in contrast to the previous findings, we did not detect *SHOX* mutations in almost half the LWS families (41%) investigated, which suggests genetic heterogeneity.

Patients, material and methods

Patients

Thirty-two individuals from 18 families with Léri-Weill syndrome were identified presenting Madelung deformity, mesomelia and short stature. Informed consent was obtained either from the participants or, in the case of children, from a parent. Families 1 to 14 were of German origin, families 15 to 18 of Dutch. Detailed clinical examination, including anthropometric measurements,[9] and extensive radiological examination of forearms and lower legs was conducted on all patients. Height measurements were transferred to SDS using the tables derived from Reiniken and van Oost[10] for the German patients and from Fredriks *et al*[11] for the Dutch patients. X-rays were analysed according to Langer[12] who defined 12 radiological criteria for the diagnosis of Madelung deformity. In order to identify the form of growth retardation, anthropometric measurements were obtained.[9] All measurements were taken in an upright position. Upper to lower segment ratio and armspan/height were calculated from the measured values. An upper/lower segment ratio of > 1 and an armspan/height ratio < 1 indicate disproportionate short stature. Forearm length and lower leg length below the 3rd percentile were interpreted as a hint of mesomelia. All female patients, as well as male patients 1C, 6B, 11B, 18A and 18B, exhibited mesomelia. Male patients 7A, 8B, 9B, 9C and 16B did not show mesomelia. The criterion for short stature was height below –1.88 standard deviation (SDS).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) of the upper limb muscles was performed on male patients 9B and 9C and on female patients 4A and 4B. For this purpose, a 1.5 tesla-scanner with a surface coil was used. T1-weighted images (SE 640/20/1; repetition time/echo time/excitations) were obtained in the axial and coronal planes. T2-weighted images (FSE 2500/80) were obtained in the axial plane.

FISH analysis

Fluorescence *in situ* hybridisation was performed using cosmids LLN0YCO3'M'11D2 (positive for DXYS14), LLNLc110P1837 (positive for DXYS129), F2cos (positive for DXYS153), LLNLc110P2410 (positive for DXYS87), LLN0YCO3'M'15D10 (positive for DXYS201), LLN0YCO3'M'34F5 (positive for DXYS131), ICRFc104G0411 (positive for DXYS 132), LLNLc110P0625 (positive for DXYS210) (all from Rao *et al*[2]), ICRFc104D0137 (positive for ANT3),[13] 3cos (positive for DXYS214) (Rappold, GA, unpublished, 1988), P1 clone ICRFP700E0978 (positive for PRKX) (corresponds to 78QD2 in Klink *et al*),[14] CEPH YAC 924F6 (positive for DXS1060, DXS6716, DXS996),[15] ICRFc104E10129 (positive for DXS6834, DXS1139),[16] BAC FBAC-4434 478I2 (positive for DXS1407, DXS1134, DXS1223, DXS7470) (Genome Systems Inc, St Louis, MO, USA), which roughly map to 0.03 Mb, 0.2 Mb, 0.35 Mb, 0.45 Mb, 0.47 Mb, 0.5 Mb, 0.53 Mb, 0.65 Mb, 1.3 Mb, 2.25 Mb, 4.6 Mb, 6 Mb, 6.5 Mb, 9 Mb from the telomere. Furthermore, a genomic 12 kb BglII fragment containing the *SHOX* exons II–VIa and cloned in pSPL3b was used.

Biotinylated cosmid, plasmid, P1, BAC or YAC DNA was hybridised to metaphase chromosomes of stimulated lymphocytes of patients as described.[17] Hybridisation signals were detected via avidin-conjugated FITC.

PCR amplification

All PCRs were carried out in a final volume of 50 µl with 100–200 ng template, 20 pmol of each primer, 200 µM dNTPs (MBI Fermentas, St Leon-Rot, Germany), 1.5 mM $MgCl_2$, 75 mM Tris/HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 5% DMSO, and 2 U of Goldstar DNA Polymerase (Eurogentec, Seraing, Belgium). Cycling was carried out in a GeneE Thermocycler (Techne) under the following conditions: long-range PCR: 93°C for 3 min, 92°C for 10 s, T_A for 30 s, 72°C for 2.5 min (for 35 cycles), and a final extension of 5 min at 72°C. Exon amplification for SSCP analysis: 93°C for 3 min, 93°C for 20 s, T_A for 20 s, 72°C for 20 s (for 35 cycles), and a final extension of 5 min at 72°C (T_A represents annealing temperature).

Oligonucleotides

For long-range PCR analysis, the following oligonucleotide primers were used: *G108 INF* 5'-CCA CAC TGA CAC CTG CTC CCT TTG-3' and *F20-12* 5'-GAA CCG CGG AGG GAT GCG GAC CCC TCT CCT TC-3' (T_A 64°C; 1.2 kb product); *ET45 INF* 5'-GGC TCT TCA CAT CTC TCT CTG CTT C-3' and *F20-12* (T_A 64°C; 1.4 kb product); *G108 INF* and *F20-02* 5'-CGG CGA TGC TGG AAT TCT TGC TGT TGC TTT TG-3' (T_A 62°C; 3.5 kb product); *Pro FOR* 5'-GCG GAG CCC GGA GAC CAG TAA TTG C-3' and *G310 INR* 5'-CCC TGG AGC

CGG CGC GCA AAG-3' (T_A 62°C; 0.5 kb product); *G310B FOR* 5'-ACG GCT TTT GTA TCC AAG TCT TTT G-3' and *92 REV* 5'-GGT GAC CTG TCT GTA TTT GC-3' (T_A 60°C; 2.8 kb product); *F20-14* 5'-GCA CCC TTG GGA GGA AGA TAT GTA TTT AAA GG-3'and *SHOX2A REV* 5'-GCC TCA GCA GCA AAG CAA GAT CCC-3' (T_A 60°C; 1.4 kb product).

For SSCP analysis, oligonucleotide primer pairs specific for exons II, III, IV, V and VI were used as described.[2] For exon II, two additional primer pairs were used: primer pair for exon II, 5' splice site: *SHOXIN1 FOR* 5'-CCT CTC TCC AGC CGT GAA CTC CTT G-3' and *SHOXEX2 REV* 5'-CAG GTT ACC CGC GTT CTC TCC GTG-3' (T_A 62°C; 98 bp product). Primer pair for exon II, 5' untranslated region: *Pro FOR* and *Pro REV* 5'-GTG CGC GCG GGT GGA TCA CCA GC-3' (T_A 60°C; 140 bp product).

Southern blot hybridisation

Southern blot hybridisations were carried out in Church buffer (0.5 M NaPi pH 7.2, 7% SDS, 1 mM EDTA) at 65°C in high stringency conditions and washed in 40 mM NaPi, 1% SDS, at 65°C.

SSCP analysis

SSCP analysis was performed on amplified genomic DNA from patients as described.[18] The PCR products (1–5 μl) were mixed with 5 μl of denaturation solution containing 95% formamide and 10 mM EDTA pH 8.0 and denatured at 95°C for 10 min. Samples were immediately chilled on ice and loaded on a 10% polyacrylamid gel (acrylamide: bisacrylamide = 37.5:1 and 29:1, multi-slot gel, TGGE base, Qiagen, Hilden, Germany) containing 2% glycerol and 1 × TBE. Gels were run at 15°C, in 1 × TBE, 500 V for 3 to 5 h and silver-stained as described.[19]

Cloning and sequencing of PCR products

PCR products were cloned into the pCR2.1–TOPO vector (Invitrogen, Groningen, Netherlands). Ten clones per exon were sequenced with Cy5-labelled vector primers M13, universal and reverse, by the cycle sequencing method described by the manufacturer (ThermoSequenase Kit, Amersham Pharmacia Biotech, Feiburg, Germany), and analysed on an ALF*express* automated sequencer (Pharmacia).

Results

Clinical findings and inheritance in patients with Léri-Weill syndrome

Bilateral Madelung deformity was present in all adult patients studied. All adults exhibited a clearly visible bayonet position of the hands ascertained by X-ray examination. Figure 1a and b show X-rays of the forearm of patients with and without *SHOX* mutation. First clinical signs of Madelung deformity such as triangulation of carpal bones and bowing of both forearm bones could be detected in children only by radiological examination. First radiological changes are already visible between 2 and 5 years of age.

Height ranged from 135 to 164 cm (–6.4 to –0.6 SDS) in adult females and from 156 to 171 cm (–3.6 to –1.8 SDS) in adult males (Table 1). Consequently short stature could not be diagnosed in two out of 18 adult females and in one out of five adult males. The SDS in five children (aged 6 to 15 years) ranged from –4.2 to –0.2 SDS (Table 1). In 14 adult patients with *SHOX* mutation, the mean height SDS was –3.4, very similar to the –3.3 SDS in nine adult patients with no detectable SHOX mutation. Mesomelia was diagnosed in all 21 female patients (adults and children) and in five out of 11 males.

Athletic habitus was obvious in male patients, except for patient 7A, and in female patients 4A and 4B. This feature was overlooked in previous clinical evaluations, but seems to be frequent in LWS. Muscle MRI revealed this habitus to be due to true muscular hypertrophy (Figure 2). An underlying muscular disorder could be excluded by normal electromyography and normal levels of creatin kinase.

Seven out of 32 patients were sporadic cases (22%), all of them females. In the remaining 25 familial cases, maternal transmission could be observed in families 1, 4, 6, 8, 11 and 17, and paternal transmission in families 7, 10, 14, 16 and 18. In family 9, maternal transmission was apparent from grandmother to father, and paternal transmission from father to son (Figure 6).

FISH analysis

To search for *SHOX* gene deletions, fluorescence *in situ* hybridisation (FISH) analysis was performed. *SHOX* containing cosmids LLN0YCO3'M'15D10 and LLN0YCO3'M'34F5, and a 12 kb BglII fragment from within cosmid 34F5 (containing *SHOX* exons II–VIa) were hybridised on metaphase chromosomes of all patients (except patient 7A, where no metaphases were available). A complete deletion of the cosmids could be detected in patients from families 1, 2, 4, 6, 11, 13, 14, 16, 17 and 18 (Figure 3).

A more detailed characterisation of the deletion breakpoints revealed six small interstitial PAR1 deletions on the X chromosome in patients 1A-D, 4A-B, 13, 14A, 16A and 18B, and on the Y chromosome in patients 14B, 16B and 18A. The deletion sizes in these families ranged from 150 kb to 450 kb (Figure 4). In contrast, families 2, 6, 11 and 17 exhibited large deletions of approximately 6 Mb and 9 Mb in size. Families 2, 6 and 11 had terminal deletions (Figure 4).

Exclusion of microdeletions by long-range PCR and Southern blot analysis

Long-range PCR was carried out to exclude microdeletions of *SHOX* in patients without detectable *SHOX* deletions. For this purpose, genomic DNA from one member of each family was investigated by long-range PCR. The amplified PCR products covered most of the *SHOX* gene region (including exon II, IV, V, VIa and VIb) (Figure 5). No specific long-range primer pairs

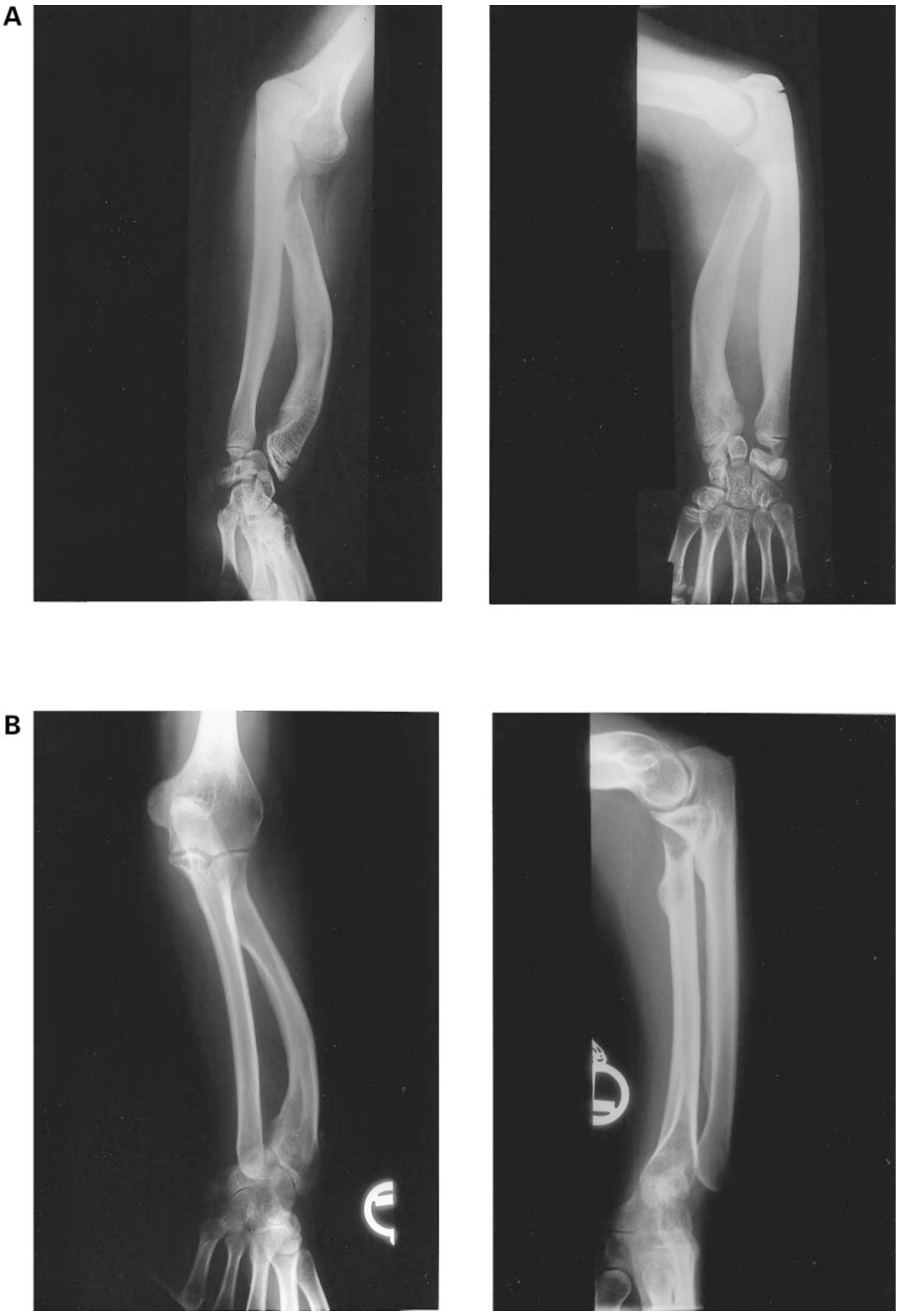

Figure 1 X-rays of the forearm, frontal and lateral view from adult patients demonstrating the classical Madelung deformity. **A**: X-rays of Madelung deformity in patient 4B with SHOX mutation. **B**: X-rays of Madelung deformity in patient 5 without detectable SHOX mutation.

Table 1 Height and approximate size of the chromosomal deletions in 32 patients from 18 different families with Léri-Weill syndrome. Three further females and three males of family 1, the mother of patient 4A, the father of patient 7A, as well as the father and brother of patient 10A, were also affected by Léri-Weill syndrome, but were not integrated in the study (Figure 6)

Patient	*Age*	*Sex*	*Height (cm)*	*SDS*	*SHOX*
1A	34	f	143	–4.7	1× (Del 450 kb)
B	31	f	150	–3.3	1× (Del 450 kb)
C	14	m	160	–0.5	1× (Del 450 kb)
D	24	f	149	–3.5	1× (Del 450 kb)
2A	17	f	135	–6.4	1× (Del 9 Mb)
B	17	f	135	–6.4	1× (Del 9 Mb)
3	29	f	164	–0.6	2×
4A	35	f	160	–1.4	1× (Del 450 kb)
B	15	f	154	–2.3	1× (Del 450 kb)
5	36	f	145	–4.3	2×
6A	31	f	147	–3.9	1× (Del 6 Mb)
B	6	m	105	–3.1	1× (Del 6 Mb)
7A	17	m	160	–2.8	no tested by FISH
8A	47	f	142	–4.9	2×
B	18	m	156	–3.6	2×
9A	60	f	148	–3.7	2×
B	35	m	164	–2.4	2×
C	7	m	110	–3.0	2×
10A	36	f	151	–2.9	2×
11A	35	f	145	–4.3	1× (Del 9 Mb)
B	6	m	86	–4.2	1× (Del 9 Mb)
12	35	f	143	–4.7	2×
13	20	f	157	–2.2	1× (Del 450 kb)
14A	18	f	152	–3.0	1× (Del 150 kb)
B	41	m	166	–2.2	1× (Del 150 kb)
15	22	f	154	–2.6	2×
16A	9	f	126	–1.9	1× (Del 450 kb)
B	37	m	171	–1.8	1× (Del 450 kb)
17A	12	f	154	–0.2	1× (Del 6 Mb)
B	37	f	153	–2.7	1× (Del 6 Mb)
18A	6	m	117	–0.6	1× (Del 450 kb)
B	44	m	167	–2.4	1× (Del 450 kb)

1× = one copy of *SHOX* is deleted; 2× = two copies of *SHOX* are present. The X-chromosomal deletion sizes are indicated in bars.

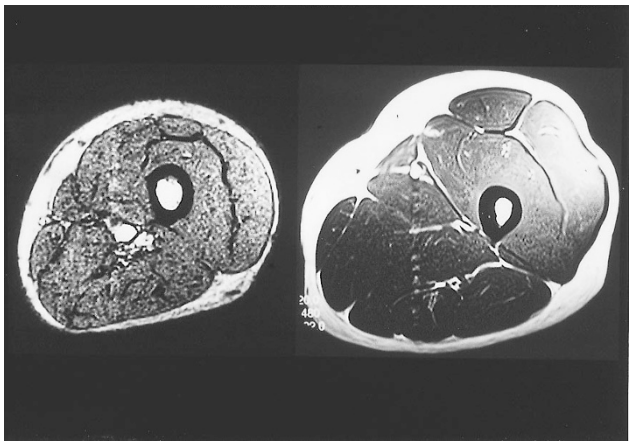

Figure 2 Muscle MRI of patient 9B on the right in comparison with an unaffected control person on the left. For patient 9B, true muscular hypertrophy is shown in the left femur (T1-weighted images were obtained in the axial planes in the middle of the diaphysis).

for exon I and III could be established due to CG-rich repeats in these regions. In addition, Southern blot analysis was performed after digestion of genomic DNA with EcoRI of patients 3, 5, 9A, 12 and 15 using a *SHOX* cDNA probe covering exon II to VIa (exon I only includes noncoding 5' UTR). Neither intronic nor exonic microdeletions were detectable by long-range PCR and genomic Southern blot analysis (data not shown).

Mutation screening and sequencing in patients with no detectable *SHOX* deletion

One patient from each family without detectable *SHOX* deletion was analysed for point mutations or small rearrangements by single-strand conformation polymorphism (SSCP) analysis. Specific (intronic) primer pairs, originally designed for the SSCP analysis of patients with idiopathic short stature,[2] were used for this purpose. In addition to these primers, two new primer pairs of the 5' splice site and the 5' untranslated region of exon II were designed. Due to an unusually high frequency of CG-rich repeats, no specific

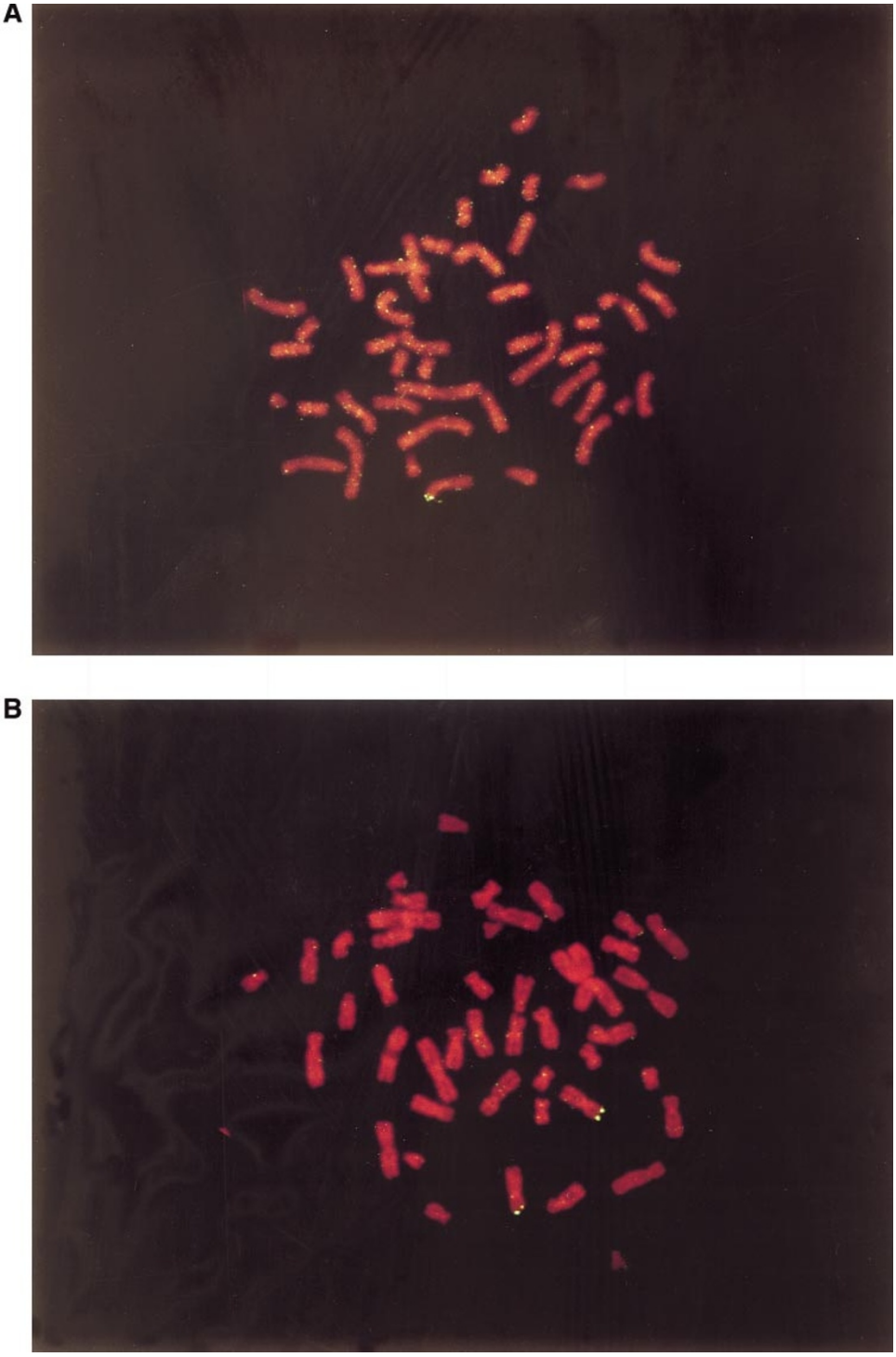

Figure 3 Fluorescence in situ hybridisation of the SHOX containing cosmid LLN0YCO3'M'34F5 to metaphase chromosomes of patient 4A (**A**) and 5 (**B**).

primers could be created for exon I, which includes part of the 5' UTR. PCR products were analysed on SSCP gels using two different conditions. No band shifts were detected. To exclude the possibility of undetected point mutations or

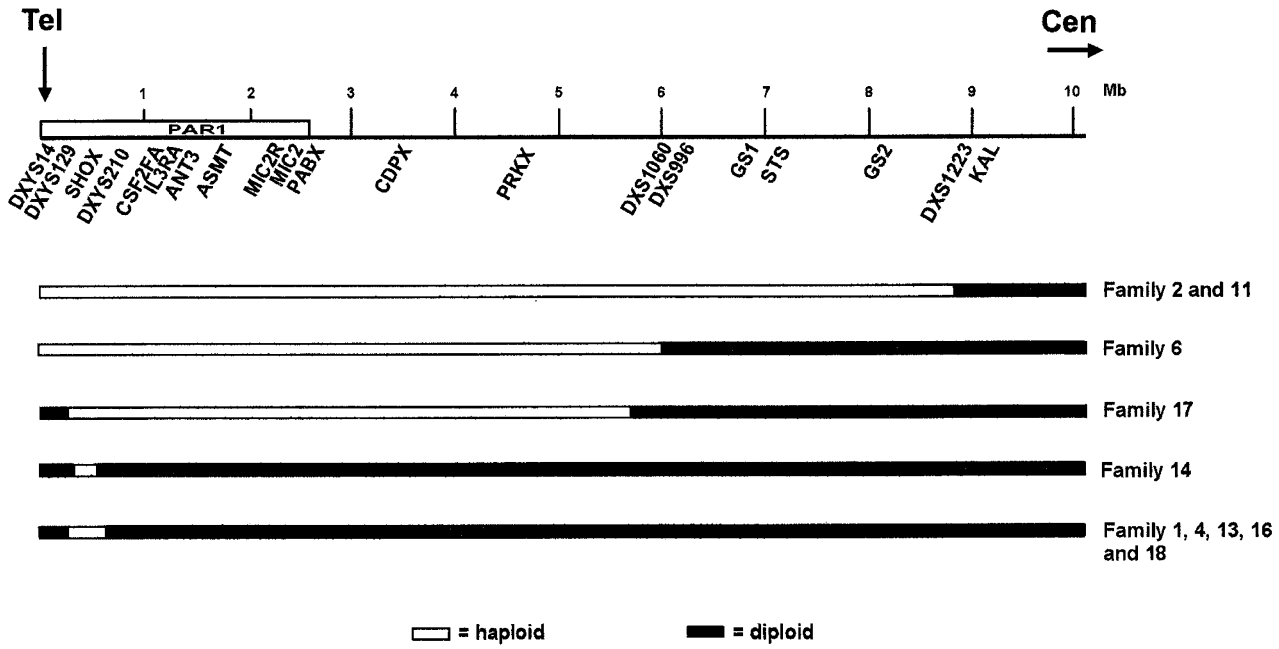

Figure 4 Schematic diagram of the chromosomal deletion sizes in 10 families. Family 14 has the smallest deletion of approximately 150 kb, and families 2 and 11 have the largest deletions encompassing roughly 9 Mb of DNA. Families 2, 6 and 11 have terminal deletions, whereas all the others presented interstitial deletions within or beyond the pseudoautosomal region. Pseudoautosomal deletions of patient 14B, 16B and 18A are on the Y chromosome, whereas the deletions of all other patients are on the X chromosome.

small rearrangements, the *SHOX* coding region was also sequenced from patients 5, 9A, 12 and 15. Sequencing did not reveal any sequence alterations.

Discussion

We identified (sub)microscopic deletions encompassing the *SHOX* gene region in 10 out of 18 families investigated. Phenotypic manifestations of LWS (short stature, mesomelia and Madelung deformity) were independent of the presence or absence of *SHOX* mutations, yet generally more severe in females. Distinct clinical features can be found independently from each other, eg patients with mesomelia were not shorter than the other patients.

There were striking variations in final height between 135 and 164 cm (–6.4 to –0.6 SDS) in females and 156–171 cm (–3.6 to –1.8 SDS) in males with a deviation into the normal range. No differences between the mean height SDS of LWS

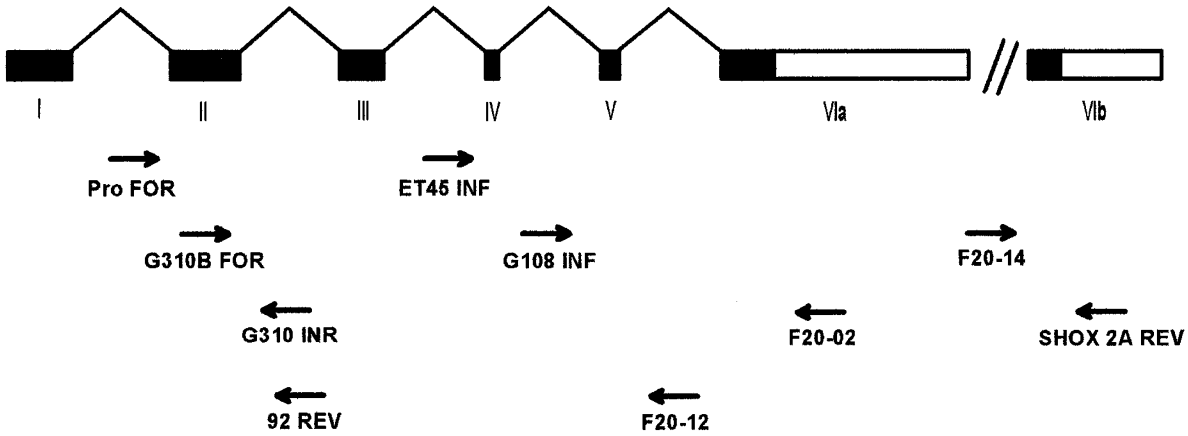

Figure 5 Schematic diagram of the genomic structure of SHOX indicating the positions of primers used for long-range PCR. Sizes of exons, introns and primers are not drawn to scale.

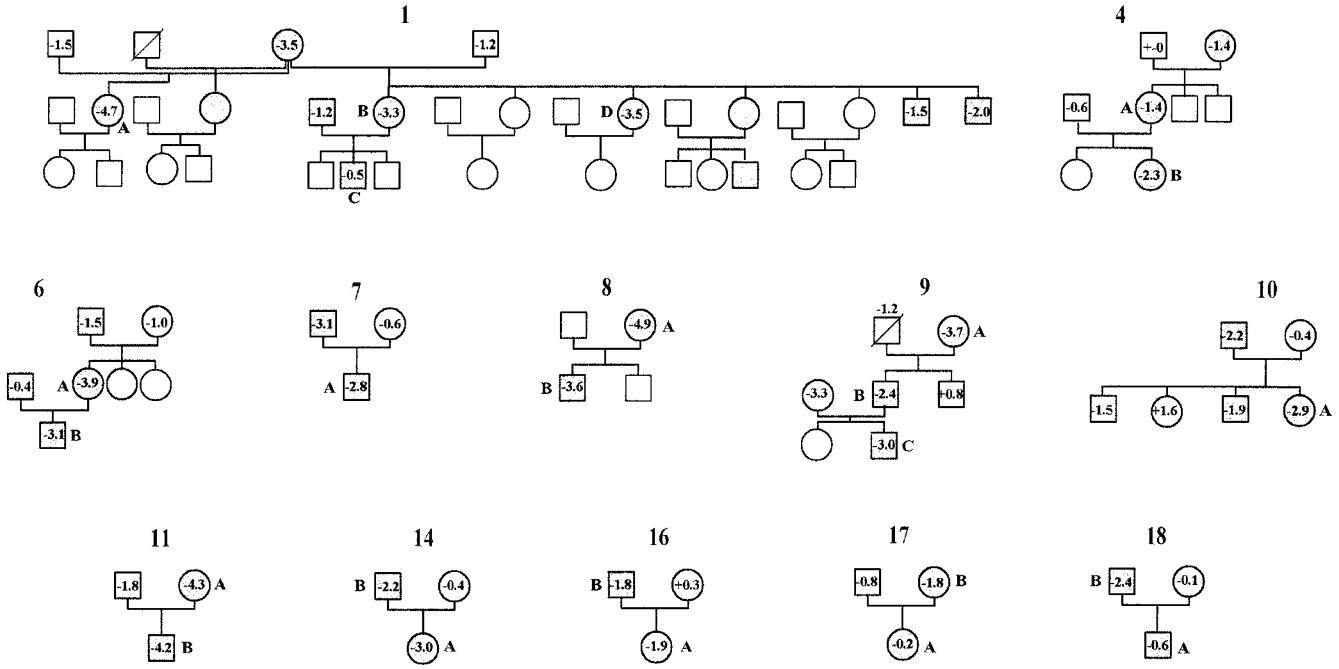

Figure 6 Pedigrees of the familial LWS cases presented in Table 1. Affected individuals with Madelung deformity are indicated by filled symbols. Unaffected individuals without Madelung deformity are represented by open symbols. Where available, height is expressed as the standard deviation (SD) score within the symbols.

patients with (–3.4 SDS) and those without *SHOX* mutation (–3.3 SDS) were evident. Striking inter- and intra-familial variations in height could be observed in the LWS patients. In family 1, for example, the SDS of height ranged from –1.5 to –4.7 in adult patients. Significant height variations have been previously described in patients with Turner syndrome[3] and hypochondroplasia.[20]

In addition, the severity of mesomelia varied, but does not seem to be an essential clinical sign of LWS, since only some of the male patients investigated (remarkably all with *SHOX* deletions) exhibited this trait. Furthermore, a variability of the manifestation of Madelung deformity was observed even within the same family (eg families 1 and 16). Phenotypic heterogeneity and intrafamilial variability are frequently found in haploinsufficiency syndromes,[21] suggesting that multiple interactions of the participating determinants are particularly sensitive to disturbance, leading to imbalance.

Phenotypic heterogeneity involving identical *SHOX* point mutations leading, for example, to proportionate short stature in otherwise normal individuals or to mesomelic short stature and Madelung deformity in Léri-Weill patients has been described previously.[2,8] Modifier genes, intragenic sequence alterations, long-range effects, epistasis, epigenetic interactions and stochastic effects have been hypothesised to explain these still unusual phenomena (for a review see Wolf[22]).

Inter- and intrafamilial variability may also be explained by the regulatory effects of sex steroids. Our clinical and molecular study has shown that muscular hypertrophy is associated with this syndrome and common in males. Recently, imprinted QTL with a major effect on muscle mass in pigs suggested the involvement of the *IGF2* gene, expressed exclusively from the paternal allele.[23] In homozygous Splotch mutant mice (carrying mutations in the homeodomain of Pax-3) a specific disturbance of muscle development in the limbs is observed.[24] In fact, Pax-3 seems to be a key regulator of somitic myogenesis, mediating activation of *MyoD*, *Myf-5* and *myogenin* in response to muscle-inducing signals.[25] The observed muscular hypertrophy in LWS males raises the possibility that *SHOX* (which shares 72% of the Pax-3 homeodomain) may in a direct or indirect way also be part of the hierarchical cascade of transcription factors regulating skeletal myogenesis. Since both alternatively spliced isoforms of *SHOX*, *SHOXa* and *b*, are expressed in skeletal muscle,[2] one may consider both gene products to be also involved in muscle development.

Madelung deformity has been diagnosed in 8% of Turner patients, constituting one of the somatic Turner signs.[4] The basic question as to why only exceptional cases of patients with *SHOX* deletions (terminal Xp/Yp deletions), *SHOX* point mutations (idiopathic short patients) and a small percentage of Turner females present Madelung deformity remains unanswered. One may argue that LWS encompasses a subset of patients where *SHOX* gene mutations phenotypically lead to the Madelung deformity, the mesomelia and short stature, and thus overlaps with a subset of Turner features.

In contrast to previous reports,[7,8] we have not detected *SHOX* mutations in almost half (41%) of our LWS families studied. There are two possible explanations for our results. First, mutations in exon I and the promoter, microdeletions of exon I and III or position effects on *SHOX* expression levels cannot be excluded. The promoter region of *SHOX* has not yet been identified, and long-range position effects have been shown to play a critical role in a number of transcription factor genes investigated.[26,27] Secondly, clinical criteria for family selection may have slightly varied in the different groups. In our study, all patients showed the bilateral Madelung deformity. A clinical re-evaluation of our patients showed no significant differences in height between Léri-Weill patients with and those without *SHOX* mutations. Moreover, all studied females presented the mesomelia, and no differences in those with or without *SHOX* mutation were found. In contrast, all five males with a mesomelia presented *SHOX* mutations, whereas four males without mesomelia showed an absence of *SHOX* mutations. This suggests that LWS is a genetically heterogeneous disease involving mutations in different genes. More extensive genome-wide linkage studies on *SHOX* positive (non-deletion) families will be required to clarify this issue. The general association of *SHOX* mutations with LWS in most cases, however, confirms the importance of this gene in short stature phenotypes.

Acknowledgements

We thank the Léri-Weill patients for providing blood samples, and A Jordan for comments. This work has been supported by the Deutsche Forschungsgemeinschaft and the Medizinische Fakultät in Heidelberg.

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