Phenotypic characterization of patients with deletions in the 3'-flanking *SHOX* region

Context : Leri-Weill dyschondrosteosis is a clinically variable skeletal dysplasia, caused by *SHOX* deletion or mutations, or a deletion of enhancer sequences in the 3'-flanking region. Recently, a 47.5 kb recurrent PAR1 deletion downstream of *SHOX* was reported, but its frequency and clinical importance are still unknown. *Objective* : This study aims to compare the clinical features of different sizes of deletions in the 3'-flanking *SHOX* region in order to determine the relevance of the regulatory sequences in this region.

Design : We collected DNA from 28 families with deletions in the 3'-PAR1 region. Clinical data were available from 23 index patients and 21 relatives.

Results : In 9 families (20 individuals) a large deletion (~200-900 kb) was found and in 19 families (35 individuals) a small deletion was demonstrated, equal to the recently described 47.5 kb PAR1 deletion. Median height SDS, sitting height/height ratio SDS and the presence of Madelung deformity in patients with the 47.5 kb deletion were not significantly different from patients with larger deletions. The index patients had a median height SDS which was slightly lower than in their affected family members (p=0.08). No significant differences were observed between male and female patients.

Conclusions : The phenotype of patients with deletions in the 3'-PAR1 region is remarkably variable. Height, sitting height/height ratio and the presence of Madelung deformity were not significantly different between patients with the 47.5 kb recurrent PAR1 deletion and those with larger deletions, suggesting that this enhancer plays an important role in *SHOX* expression.

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Introduction

Leri-Weill dyschondrosteosis (LWD) is caused by haploinsufficiency of SHOX in 60-70% of cases (intragenic deletions, duplications or mutations), and in 15-31% of patients without an intragenic mutation or deletion a putative enhancer sequence in the 3'-flanking region has been demonstrated(Benito-Sanz *et al.* 2005; Chen *et al.* 2009). *SHOX* is located on the short arm of the X-chromosome in the pseudoautosomal (PAR) 1 region, thereby escaping X-inactivation, leading to a pseudoautosomal pattern of inheritance for LWD (Belin *et al.* 1998, Shears *et al.* 1998). So far, 7 enhancer sequences, 3 upstream and 4 downstream of *SHOX*, have been described (Fukami *et al.* 2006; Sabherwal *et al.* 2007; Durand *et al.* 2010; Kenyon *et al.* 2011; Benito-Sanz *et al.* 2012a; Benito-Sanz *et al.* 2012b). Deletion of one or more of the downstream enhancer sequences results in SHOX haploinsufficiency and LWD. Benito-Sanz *et al.* (Benito-Sanz *et al.* 2005) as well as Chen *et al.* (Chen *et al.* 2009) described a cohort of patients with different deletions in the downstream enhancer region, where various deletion sizes all resulted in LWD or idiopathic short stature. A deletion in the enhancer region upstream of *SHOX* has only recently been reported in one female patient and her father with idiopathic short stature (Benito-Sanz *et al.* 2012a), where two of the three upstream enhancer sequences were deleted.

LWD caused by a heterozygous mutation or deletion of *SHOX* is characterized by short stature, mesomelia, and Madelung deformity. Penetrance of the phenotype appears to be incomplete within families (Rappold *et al.* 2007; Binder, 2011) and Madelung deformity and short stature tend to be more common and severe in females than in males (Binder, 2011). Mean height of index cases has been reported as -2.2 SDS (Binder, 2011; Rosilio *et al.* 2012) and height of affected mothers and fathers as -2.1 SDS and -1.6 SDS, respectively (Rosilio *et al.* 2012).

42 There is limited information about the phenotype of patients with a deletion in the enhancer

43 region downstream of *SHOX*. However, from the available reports the phenotypic

44 characteristics do not seem to differ much from those of patients with *SHOX* deletions or

45 mutations (Benito-Sanz *et al.* 2005, Salmon-Musial *et al.* 2011, Rosilio *et al.* 2012).

46 Recently, a relatively small deletion of PAR1 was found by Caliebe *et al.* (Caliebe *et al.* 2012)

47 in a father and son, of whom the son had short stature (-2.0 SDS), while the father did not

48 (-0.44 SDS). Comparable deletions were already demonstrated by Chen *et al.* (Chen *et al.*

49 2009), but their exact size was not determined in detail. Subsequently this deletion was

50 described in 30 unrelated families with LWD or ISS by Benito-Sanz *et al.* (Benito-Sanz *et al.*

51 2012b) equivalent to 15.3% of probands with LWD and 1.9% of probands with idiopathic

52 short stature, uncovering a novel downstream enhancer. The clinical phenotype of these

53 patients showed a wide variability with height SDS ranging from -0.14 to -4.68.

54 We aimed at investigating the frequency of the various deletions in the 3'-flanking *SHOX*

55 region and the possible phenotypic differences between patients with different sizes of

56 deletions in this region.

Methods

Clinical characteristics

In 49 DNA samples sent to the Laboratory for Diagnostic Genome Analysis of the Department of Clinical Genetics (Leiden University Medical Center, Leiden) and in six of the DNA samples sent to the Section Genome Diagnostics of the Department of Genetics (University Medical Center Groningen, Groningen), for the determination of *SHOX* defects between 2005 and 2011, we found a deletion in the downstream enhancer region of *SHOX* in 29 probands, and 26 of their relatives (total of 28 families). In the same period in the Laboratory for Diagnostic Genome Analysis of the Department of Clinical Genetics (Leiden University Medical Center, Leiden) we detected 43 deletions of the complete gene, 22 mutations (missense, nonsense, splice site or frameshift) and 5 duplications of *SHOX* in a total of 1045 patients.

With consent of the Medical Ethical Committee of the LUMC, clinical data were collected and anonymized for 44 of the 55 patients (23 index patients and 21 affected relatives from 23 families). Two of the remaining 11 patients were excluded from the analysis of clinical features, because they suffered from an additional disorder, which would probably influence their growth (a skeletal dysplasia of unknown type (inconsistent with LWD), and a deletion 22q11, respectively). For 9 patients no additional clinical data could be obtained.

Demographic and clinical data collected were sex, age, height standard deviation score (SDS), sitting height to height ratio SDS and presence of Madelung deformity. Height SDS and sitting height/height SDS were calculated based on Dutch nation-wide references (Fredriks *et al.* 2005). We chose not to collect all data needed to calculate the Rappold score (Rappold *et al.* 2007), because of the limitations of this scoring system in clinical practice: arm span, and length and bowing of the forearm are seldom measured by clinicians, appearance of muscular

81 build is not very often spontaneously mentioned in clinical records, and the use of body mass

82 index gives a higher score for obese patients. Instead, we chose to collect data about the

83 classical clinical triad of Leri-Weill dyschondrosteosis: height, sitting height to height ratio

84 (SH/H) and presence of Madelung deformity.

85

86 *Molecular analysis*

87 DNA isolation from peripheral blood and sequence analysis of the *SHOX* coding region

88 including intron-exon boundaries was performed using standard procedures (primer

89 sequences available upon request). Deletions and duplications were detected using the

90 MRC-Holland MLPA kit (Salsa P018-D1) according to the manufacturer's instructions.

91

92 *Statistical analysis*

93 The t-test and Fisher exact test were used to test for an association of genotype (deletion

94 including PAR1 probes L05099-L05101 or larger deletions) with height SDS, SH/H SDS, and

95 the presence of a Madelung deformity. Differences between index patients and their relatives

96 were determined, as well as the influence of the gender of the patient.

97 **Results**

98 Of the 28 families, 19 had the recurrent 47543 bp (47.5 kb) deletion encompassing PAR1

99 probes L05099-L05101 described by Benito-Sanz *et al.* (Benito-Sanz *et al.* 2012b),

100 containing a *SHOX* enhancer sequence (ECR1/CNE7). The deletion was found in 19 index

101 cases and 16 of their relatives. Nine families (10 index patients and 10 relatives) had a larger

102 deletion varying from a deletion that includes PAR1 probes L05096-L05104 to a deletion

103 including PAR1 probes L05098-L05103 (figure 1).

104 In the 44 individuals (23 families) of whom clinical information was available, mean height

105 SDS was -1.9 (range -4.0 to 1.2) and SH/H SDS 1.5 (range -1.6 to 3.0). Madelung deformity

106 was present in 19 of 33 patients. Mean height SDS and SH/H SDS in patients with the 47.5 kb

107 deletions (n=25) compared to patients with larger deletions (n=19) are shown in figure 2.

108 Patients with the 47.5 kb deletion were on average 0.5 SD shorter than patients with larger

109 deletions, but the difference did not reach statistical significance (p=0.20). A comparison of

110 the same clinical characteristics between the index patients and their affected relatives, and

111 between male and female patients, is also shown in figure 2. No significant differences were

112 observed in the different subgroups of patients, as illustrated in figures 2 and 3. Presence or

113 absence of a Madelung deformity in the subgroups of patients mentioned above is

114 summarized in table 1. No significant differences were observed in any of the subgroups.

Discussion

115

116 We show that the recently reported recurrent 47543 bp (47.5 kb) deletion (Benito-Sanz *et al.*

117 2012b) could also be demonstrated in 19 out of 28 Dutch families (68%) with a downstream

118 deletion and has a similar clinical phenotype as larger deletions in the downstream region of

119 *SHOX*. Height SDS, SH/H SDS and the presence of Madelung deformity all showed a large

120 variation, regardless of deletion size, gender of the patient or whether the patient was an index

121 patient or relative.

122 We have now shown that these deletions comprise an important part of our patient cohort. The

123 lack of previous reports on this deletion could be due to doubt about the pathogenity of the

124 deletion, because it is not overlapping the formerly described critical region in the enhancer

125 region (Benito-Sanz *et al.* 2005). However, recently the same research group convincingly

126 reported this deletion to include a previously uncharacterized *SHOX* enhancer (ECR1/CNE7),

127 proving the deletion to be pathogenic (Benito-Sanz *et al.* 2012b).

128 The average height SDS in our index cases (-2.2 SDS) is similar to mean height SDS in

129 cohorts of patients with *SHOX* deletions and mutations (Binder, 2011). In the first report on

130 patients with a deletion in the enhancer region Benito-Sanz *et al.* (Benito-Sanz *et al.* 2005)

131 demonstrated 12 families with short stature and/or Madelung deformity with variable severity,

132 with a height SDS ranging from -4.6 SD to 0.85 SD. Rosilio *et al.* (Rosilio *et al.* 2012)

133 recently reported a mean height in their patients with a deletion encompassing *SHOX* of -2.3

134 SDS, while in the same study a mean height of -2.2 SDS was seen in patients with any

135 deletion in the downstream region. In a smaller sample of patients with a deletion downstream

136 of *SHOX* in the study of Salmon-Musial *et al.* (Salmon-Musial *et al.* 2011) a mean height

137 SDS of -1.4 SDS was demonstrated. The results of these reports are in accordance with our

138 observations, although in the two last mentioned reports it is suggested that the phenotype in

139 patients with a deletion in the downstream enhancer region seems to be milder than in patients

140 with a *SHOX* mutation or deletion. The six patients described by Salmon-Musial *et al.*

141 (Salmon-Musial *et al.* 2011) with anomalies downstream of *SHOX* had a less severe short

142 stature (height SDS -1.4) than those with other gene anomalies (height SDS -2.5; n=16). Due

143 to the small size of their sample they were not able to draw firm conclusions. Rosilio *et al.*

144 (Rosilio *et al.* 2012) reported a higher percentage of idiopathic short stature (instead of LWD)

145 in patients with a deletion in the downstream enhancer region interval compared to patients

146 with a *SHOX* mutation or deletion, suggesting a milder phenotype in patients with a

147 downstream deletion. However, classification of patient groups in this study shows some

148 shortcomings, as also acknowledged by the authors, and comparisons must be taken with

149 caution. Furthermore in contrast to the findings of Rosilio *et al.* (Rosilio *et al.* 2012), Chen *et*

150 *al.* (Chen *et al.* 2009) reported a higher percentage of downstream deletions in LWD patients

151 compared to patients with idiopathic short stature. Therefore, genotype-phenotype

152 correlations do not seem obvious.

153 Comparing all index patients with their relatives shows that mean height SDS is slightly

154 higher in relatives, although not significantly different (p=0.08), probably due to referral bias.

155 Differences between male and female patients were not observed in our study, contrary to

156 what is known about patients with *SHOX* deletions or mutations where females tend to

157 present with a more severe phenotype than males (Binder, 2011). However, further

158 confirmation is needed on a larger patient cohort.

159 The large variability of the phenotype confirms the findings in previous reports on patients

160 with a deletion in the enhancer region 3'of *SHOX* (Benito-Sanz *et al.* 2005; Chen *et al.* 2009;

161 Kant *et al.* 2011; Salmon-Musial *et al.* 2011; Rosilio *et al.* 2012). Not all index patients had

162 short stature, which was also noticed in the study by Salmon-Musal *et al.* (Salmon-Musial *et*

163 *al.* 2011), and has been observed in previous studies (Binder *et al.* 2004, Rappold *et al.* 2007).

164 Looking in detail at this group of eight patients with a height at last examination above -2

165 SDS, reason for a diagnostic *SHOX* analysis was the presence of Madelung deformity in five

166 of them, and disproportion in combination with familial disproportionate short stature in one

167 patient. Another patient had a recent height measurement of -1.4 SDS, but former height

168 measurements were always around -2 SD, while several family members had disproportionate

169 short stature. The last patient had a height in the normal range, but below her target height

170 range. So, short stature is not always the leading clinical sign to request *SHOX* analysis, and

171 referring clinicians do take into account other clinical characteristics of the patient and their

172 relatives. The variability of the clinical phenotype is not limited to deletions of the enhancer

173 region. Also for LWD caused by *SHOX* mutations or deletions, the phenotype has been

174 described as highly variable (Binder, 2011). Thus, penetrance of the phenotype appears to be

175 incomplete, even within families.

176 In conclusion, the phenotype in patients with a deletion in the downstream enhancer region of

177 *SHOX* seems comparable to that of patients with a *SHOX* mutation or deletion, and the

178 variability of the phenotype in the whole group of patients with a deletion in the enhancer

179 region is remarkable. The recurrent 47.5 kb deletion downstream of *SHOX* leads to a

180 phenotype comparable to that of larger deletions in the same region. Our data support that the

181 47.5 kb deletion is a pathogenic deletion, and therefore it seems plausible that an important

182 enhancer region is located within the deletion interval.**Acknowledgements**

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Figure 1

Schematic representation of the SHOX locus and the surrounding enhancer regions

Schematic representation of the *SHOX* locus and location of the 3'-flanking PAR1 probes of the P018-D1 MLPA kit (MRC Holland). Extent of the deletions (blue bars) and the number of families (n) is indicated with the number of affected individuals between brackets. The SHOX gene is indicated with a purple box. The PAR1 probes are indicated with an * and the four downstream enhancers (ECN4, ECN5, ECR1/CNE7 and CNE9) are indicated with green or red (the enhancer within the 47.5 deletion) triangles. The deletion described by Sabherwal includes PAR1 probes L05096-l L05101.

Figure 1

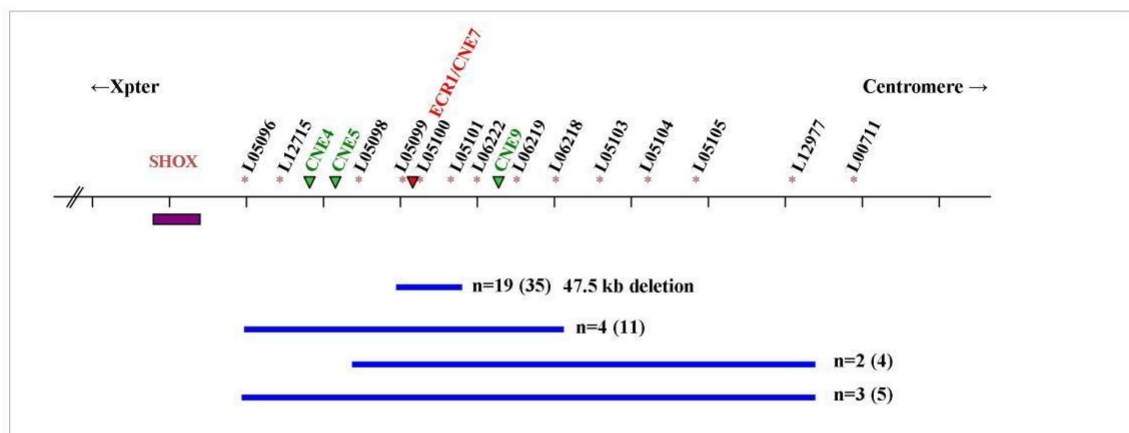



Figure 2

Height (SDS) range in different subgroups of patients

Height (SDS) range in different subgroups of patients with a deletion in the downstream enhancer region of *SHOX*. del 47.5 kb = deletion that includes PAR1 probes L05099-L05101 in the downstream enhancer of *SHOX*; other del = larger deletion in the downstream enhancer of *SHOX*; *=mean value; n=number of tested individuals; p=p-value measured by t-test.

Figure 2.

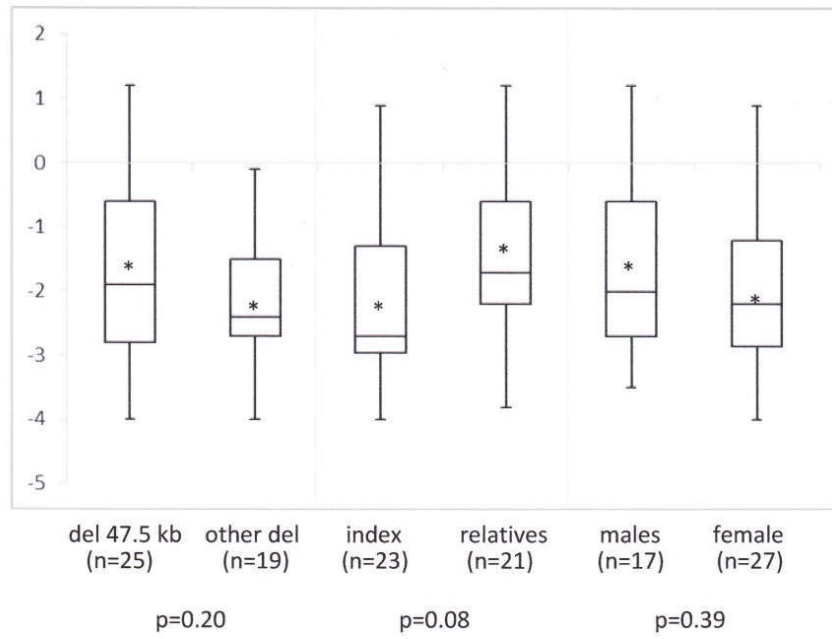
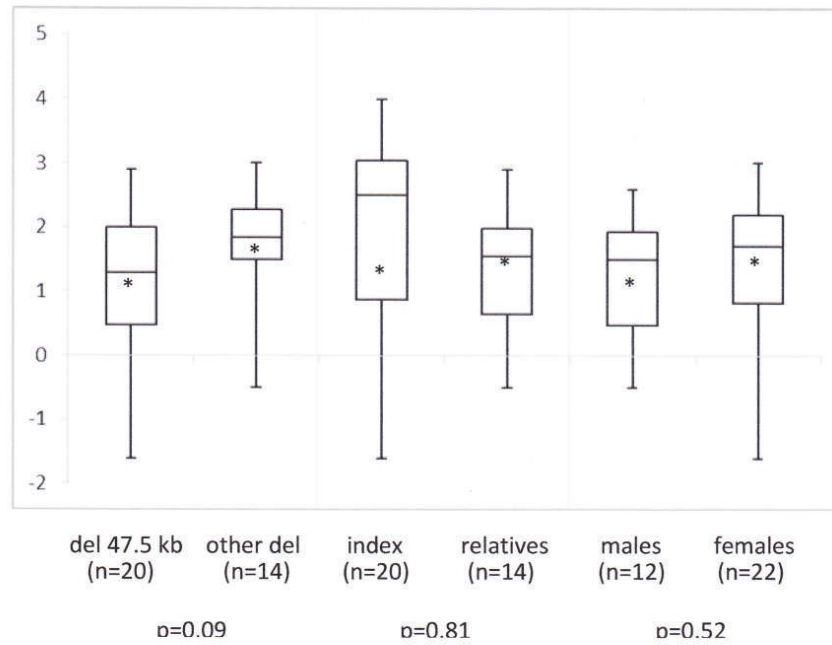



Figure 3

Sitting height to height ratio (SDS) range in different subgroups of patients

Sitting height to height ratio (SDS) range in different subgroups of patients with a deletion in the downstream enhancer region of *SHOX*. del 47.5 kb = deletion that includes PAR1 probes L05099-L05101 in the downstream enhancer of *SHOX*; other del = larger deletion in the downstream enhancer of *SHOX*; *=mean value; n=number of tested individuals; p=p-value measured by t-test.

Figure 3.

Madelung deformity in different subgroups of patients with a deletion in the downstream enhancer region of SHOX

Three comparisons are made in subgroups of patients. del 47.5 kb = deletion that includes PAR1 probes L05099-L05101 in the downstream enhancer of *SHOX*; other del = larger deletion in the downstream enhancer of *SHOX*.

Table 1.

	Madelung present	Madelung absent	p-value (Fisher exact test)
patients with del 47.5 kb (n=17)	11	6	0.49
patients with other del (n=16)	8	8	
index patients (n= 19)	10	9	0.72
relatives (n=14)	9	5	
male patients (n= 12)	7	5	0.72
female patients (n=21)	14	7	