

ARTICLE

The disruption of a novel limb cis-regulatory element of *SHH* is associated with autosomal dominant preaxial polydactyly-hypertrichosis

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The expression gradient of the morphogen *Sonic Hedgehog* (*SHH*) is crucial in establishing the number and the identity of the digits during anteroposterior patterning of the limb. Its anterior ectopic expression is responsible for preaxial polydactyly (PPD). Most of these malformations are due to the gain-of-function of the Zone of Polarizing Activity Regulatory Sequence, the only limb-specific enhancer of *SHH* known to date. We report a family affected with a novel condition associating PPD and hypertrichosis of the upper back, following an autosomal dominant mode of inheritance. This phenotype is consistent with deregulation of *SHH* expression during limb and follicle development. In affected members, we identified a 2 kb deletion located ~240 kb upstream from the *SHH* promoter. The deleted sequence is capable of repressing the transcriptional activity of the *SHH* promoter *in vitro*, consistent with a silencer activity. We hypothesize that the deletion of this silencer could be responsible for *SHH* deregulation during development, leading to a PPD-hypertrichosis phenotype.

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INTRODUCTION

Anteroposterior patterning of the limb is initiated during early development to assign the number and the identity of the digits. The zone of polarizing activity (ZPA) is a mesenchymatous region of the posterior margin and is recognized as center for this signaling pathway. ZPA cells express the secreted morphogen *Sonic Hedgehog* (*SHH*, OMIM#600725), encoding a signal protein. Its gradient of expression determines the differentiation of the digits.¹ The anterior ectopic expression of *SHH* is responsible for congenital limb malformations, in particular preaxial polydactyly (PPD).^{2–6} PPD has an incidence of ~1/2000 births. Most PPDs are due to the gain-of-function (by pathogenic variant or copy number gain) of a *SHH* cis-regulatory element. This element, the ZPA regulatory sequence (ZRS, OMIM#605522), has an enhancer activity specific for the developing bud (for review, see ref. 7). This enhancer is located ~1 Mb upstream of *SHH* (7q36). This distance can slightly differ among vertebrate species while the ordering of genes and regulatory elements is highly conserved, therefore probably having a crucial role in *SHH* regulation. The existence of other cis-regulatory elements of *SHH* specific for limb development is likely, as numerous PPD families linked to the 7q36 region do not have a ZRS alteration.^{8,9}

We studied a large family affected with autosomal dominant PPD. Limb malformations were associated with hypertrichosis affecting the upper back. This phenotypic association has not, to our knowledge, been previously reported and we hypothesize that this could be due to *SHH* deregulation. We first confirmed the linkage at the 7q36 locus in

this family, performing haplotyping with SNP-arrays. ZRS sequencing and copy number analysis was normal. Sequencing of ECRs among the candidate locus did not reveal any pathogenic variant. We then identified a 2 kb deletion at long distance upstream of *SHH*, comprising a silencer. Deletion of this regulatory element could lead to anterior overexpression of *SHH*, therefore causing PPD. Given the role of *SHH* during follicle morphogenesis and growth,^{10–12} it is likely that its deregulation is also responsible for the hypertrichosis in this family.

MATERIAL AND METHODS

Patients

Through the Clinical Genetics department of Lille University Hospital, France, we recruited a large family of European ancestry comprising 25 index cases in five generations (pedigree, Figure 1) presenting with PPD and upper back hypertrichosis, following an autosomal dominant mode of inheritance. Phenotypic expressivity was variable among individuals, ranging from triphalangeal thumbs, duplicated thumbs, preaxial extra ray to syndactyly between digits I and II (Table 1, Figure 2, Supplementary Figures 1 and 2). All affected members presented with hirsutism, starting as a low posterior hairline and spreading down to the middle of the back (Table 1, Figure 2, Supplementary Figure 3). Written patient consents were obtained prior to the analyses. Standard karyotype was normal in some affected individuals that were tested.

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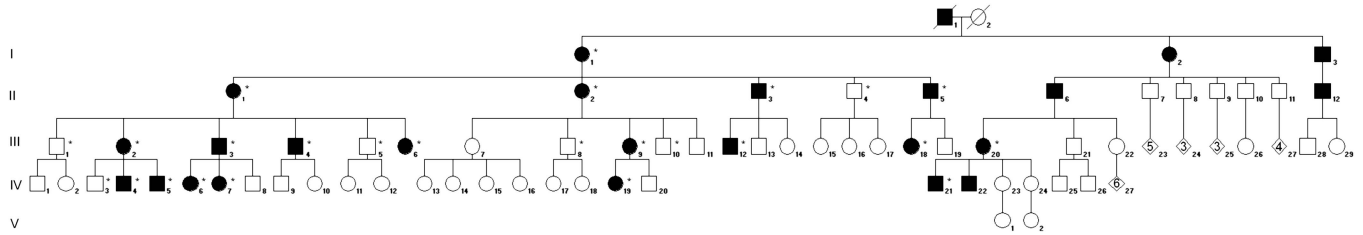


Figure 1 Pedigree of the family affected with autosomal dominant preaxial polydactyly and upper back hypertrichosis. Individuals available for phenotyping and genotyping are indicated with an asterisk.

Array-CGH

We performed array-CGH 44 K (Agilent Technologies, Courtaboeuf, France) in one affected member of the family (IV-21), to search for copy number variation, according to the manufacturer's protocol. Male genomic DNA was used as a reference in sex-match hybridization and results were analyzed with the CGH analytics software.

ZRS screening

ZRS Sanger sequencing was performed in affected members of the family using three couples of primers (primers' sequences are provided in Supplementary Table 1) to study the whole ZRS region (chr7.hg19: g.156,583,690-156,584,790). The sequence was compared with the reference sequence (Ref Seq, hg19, NC_000007.14) using SeqScape software.

To assess ZRS copy number variations, we performed a quantitative PCR assay with two primers' couples (primers' sequences are provided in Supplementary Table 1) in affected individuals. The reaction was performed with the SYBR Green technology according to the manufacturer's protocol (Applied Biosystems). Quantification of the target sequences was normalized to an assay from *RPPH1* gene, and the relative copy number was determined on the basis of the comparative $\Delta\Delta Ct$ method using a normal control DNA as the calibrator.

Haplotyping by SNP-arrays

We performed SNP-arrays (CytoSNP-12 v2.1, Illumina, San Diego, USA) in 16 family members (I-1, II-1, II-2, II-3, II-3's partner, III-2, III-3, III-9, III-12, III-20, IV-3, IV-4, IV-5, IV-7, IV-19 and IV-21). Samples were processed according to the manufacturer's protocol, and results were analyzed using Illumina GenomeStudio software. Lod-scores were calculated using Merlin software¹³ with the following parameters: autosomal dominant model, 100% penetrance, frequency of disease 0.0001, rate of *de novo* mutation 0.0001 in both sexes.

Evolutionary conserved regions study

To identify the evolutionary conserved regions (ECRs) comprised in the candidate locus, we performed human genomic sequence alignment with the orthologous sequences of mice and chicken using the browser <http://ecrbrowser.dcode.org> with the following parameters: minimal ECR length 350 bp, minimal ECR similarity 77% (threshold defined in¹⁴). The ECRs were sequenced in an affected individual using a custom capture design SureSelect (Agilent Technologies) on the MiSeq (Illumina), according to the manufacturers' protocols. The design was performed with the SureDesign software and variant calling with the SureCall software (Agilent Technologies).

Plasmid constructs

Constructs were generated by inserting target sequence into the pLS_Prom_SHH vector (pLightSwitch_Prom_SHH vector, #S716462,

Active Motif, La Hulpe, Belgium). This plasmid contains a minimal *SHH* promoter and the Renilla luciferase reporter gene. The 2026 bp sequence of interest was obtained by PCR on genomic DNA and cloned upstream of the *SHH* promoter in the pLS_Prom_SHH vector using the MluI restriction site (primers' sequences are provided in Supplementary Table 1). Two constructs were generated, with forward (pLS_Prom_SHH_2026F) and reverse (pLS_Prom_SHH_2026R) orientation of the 2026 bp sequence.

As controls, we used the pLS_Prom_SHH vector and the pLS_EmptyProm (pLightSwitch_EmptyProm, #S790005, Active Motif). The latter does not contain any promoter upstream of the Renilla luciferase reporter gene and was used as a measure of background signal in the experiment.

To normalize with the transfection efficiency, we performed co-transfections with the pGL3 Promoter Vector (#E1761, Promega, Charbonnières, France) that contains a SV40 promoter and the Firefly luciferase reporter gene.

Cell culture and luciferase assay

Cells of the human colon adenocarcinoma cell line Caco2 were maintained in 20% FBS/MEM containing 110 mg/l sodium pyruvate, 0.1 mM non-essential amino acids, 1% glutamine and 0.5% of penicillin-streptomycin. Cells at 60–70% confluence were transfected with 0.4 μ g Renilla luciferase reporter constructs and 50 ng Firefly luciferase reporter plasmid using the Effectene transfection reagent (Qiagen, Courtaboeuf, France) in six-well plates, according to the manufacturer's protocol. After 48 h of transfection, cells were washed twice with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Renilla and Firefly luciferase reporter activities were assessed with the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's protocol, using the Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Thoiry, France). The Renilla/Firefly luciferase activity ratio for each construct was compared with the pLS_Prom_SHH construct. The transfections were performed in triplicate, three times.

RESULTS

ZRS study is normal in PPD-hypertrichosis

ZRS Sanger sequencing in affected individuals of the family revealed no pathogenic variant and quantitative PCR assay showed two copies of the limb-specific enhancer (data not shown).

PPD-hypertrichosis is linked to the 7q36 locus

No copy number variation was identified in the family by array-CGH 44 K (mean resolution 150 kb). To map the PPD-hypertrichosis condition, we performed whole genome haplotyping by SNP-arrays in 16 individuals from the family. Results showed linkage to a single locus at 7q36 (Lod-score > 3). There was no other locus in the genome with a positive Lod-score (Supplementary Figure 4). The candidate

Table 1 Clinical summary of patients affected with PPD-hypertrichosis

Patients	I-1	II-1	II-2	II-3	II-5	III-2	III-3	III-4	III-6	III-9	III-12	III-18	III-20	IV-4	IV-5	IV-6	IV-7	IV-19	IV-21	Total (% limb)
Upper limb malformations																				
Triphalangeal thumb	+/+	-/+	-/-	-/-	+/+	-/-	+/+	-/+	-/-	-/-	+/+	+/+	-/-	-/-	-/-	+/+	+/+	-/-	-/-	16/38 (42%)
Biphalaengeal partly duplicated thumb	-/-	+/+	-/-	-/-	-/-	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-	-/-	-/-	10/38 (26%)
Preaxial extra ray	-/-	-/-	+/+	-/-	-/-	-/-	-/-	+/+	-/-	+/+	-/-	-/-	-/-	-/-	-/-	+/+	+/+	+/+	-/+	11/38 (29%)
Syndactyly digits I-II	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	ND	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	-/-	7/36 (19%)
Lower limb malformations																				
Large hallux	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	ND	-/+	-/+	+/+	+/+	-/-	-/-	+/+	+/+	-/-	+/+	24/36 (67%)
Duplicated hallux	-/-	+/+	+/+	-/-	-/-	+/+	+/+	-/-	+/+	-/-	-/-	-/-	+/+	-/-	-/-	+/+	+/+	+/+	+/+	19/38 (50%)
Syndactyly toes I-II	-/-	+/+	+/+	-/+	-/-	+/+	-/-	-/-	ND	+/+	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-	-/-	13/36 (36%)
Upper back hypertrichosis	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	18/18 (100%)

Abbreviation: ND, not determined.

region spans 2.1 Mb between rs2305944 and rs4101 (chr7.hg19:g.155809123_157947033), containing seven genes, seven non-coding RNAs and one micro-RNA (Figure 3). It is located upstream of the *SHH* gene and encompasses its limb-specific enhancer, the ZRS.

The candidate locus contains several ECRs

The phenotypic association of PPD and hypertrichosis is highly suggestive of *SHH* deregulation. Given that the ZRS study (sequencing and quantitative PCR) was normal in this family, we hypothesized that another *SHH* regulatory element located at 7q36 could be disrupted. In this hypothesis, we identified 66 ECRs (including the ZRS) in the candidate locus of 2.1 Mb (Supplementary Table 2). Some of these have already been described and studied in PPD families.^{8,9} Sequencing of these regions in one individual affected with PPD-hypertrichosis (IV-21) revealed no pathogenic variant.

Identification of a 2 kb deletion segregating with the phenotype

The data generated by SNP-arrays in the candidate region of 2.1 Mb were reanalysed for copy number variation. We identified a heterozygous deletion of rs4296934 located in the gene desert upstream of *SHH*, at ~240 kb from its promoter (Figure 3). This deletion was confirmed by long-range PCR-sequencing (Figure 3) and spans 2026 bp (chr7.hg19:g.155845627_155847652). This deletion segregates with the phenotype in the family with complete penetrance. The deletion was absent from 100 control chromosomes. The data were submitted in the Decipher database (<https://decipher.sanger.ac.uk/#296668>). No identical copy number variation was reported in this database. We therefore hypothesize that this deletion could be responsible for the PPD-hypertrichosis phenotype by the deregulation of *SHH* expression.

Structure of the 2 kb sequence

After mapping the PPD-hypertrichosis phenotype to the 7q36 locus, we identified a 2026 bp region deleted in the family. Inter-species alignments show that the wild-type sequence is highly conserved between human and rhesus primate, and partially conserved in mouse, dog, chicken and zebrafish (Supplementary Figure 4). In the less conserved parts of the region, three repetitive elements are located: one DNA repeat (Mer112) of 113 bp, one simple repeat of 76 bp and one *Alu* sequence of 298 bp (Supplementary Figure 5). Besides, the 2026 bp sequence contains numerous predicted binding sites for transcription factors (MatInspector, Genomatix). We selected the sites for which the matrix similarity was over 95% and filtered the transcription factors for which less than three binding sites were predicted. With these criteria, 28 transcription factors have three or more predicted binding sites on the sequence (Supplementary Table 3). Regarding these data, we hypothesize that this region could possibly contain a regulatory element.

The 2 kb deletion contains a cis-regulatory element

To elucidate a possible regulatory effect of the 2026 bp element, a functional study was initiated in cell cultures. The 2026 bp sequence was subcloned into the expression vector pLS_Prom_SHH. As the human colon adenocarcinoma Caco2 cell line expresses *SHH*, we therefore performed transfections in these cells with the construct containing the 2026 bp sequence in forward (pLS_Prom_SHH_2026F) and reverse orientation (pLS_Prom_SHH_2026R). Both constructs show an approximately twofold decrease of the relative reporter activity, as compared with the control vector without regulatory element pLS_Prom_SHH (Figure 4). Thus, the 2026 bp sequence

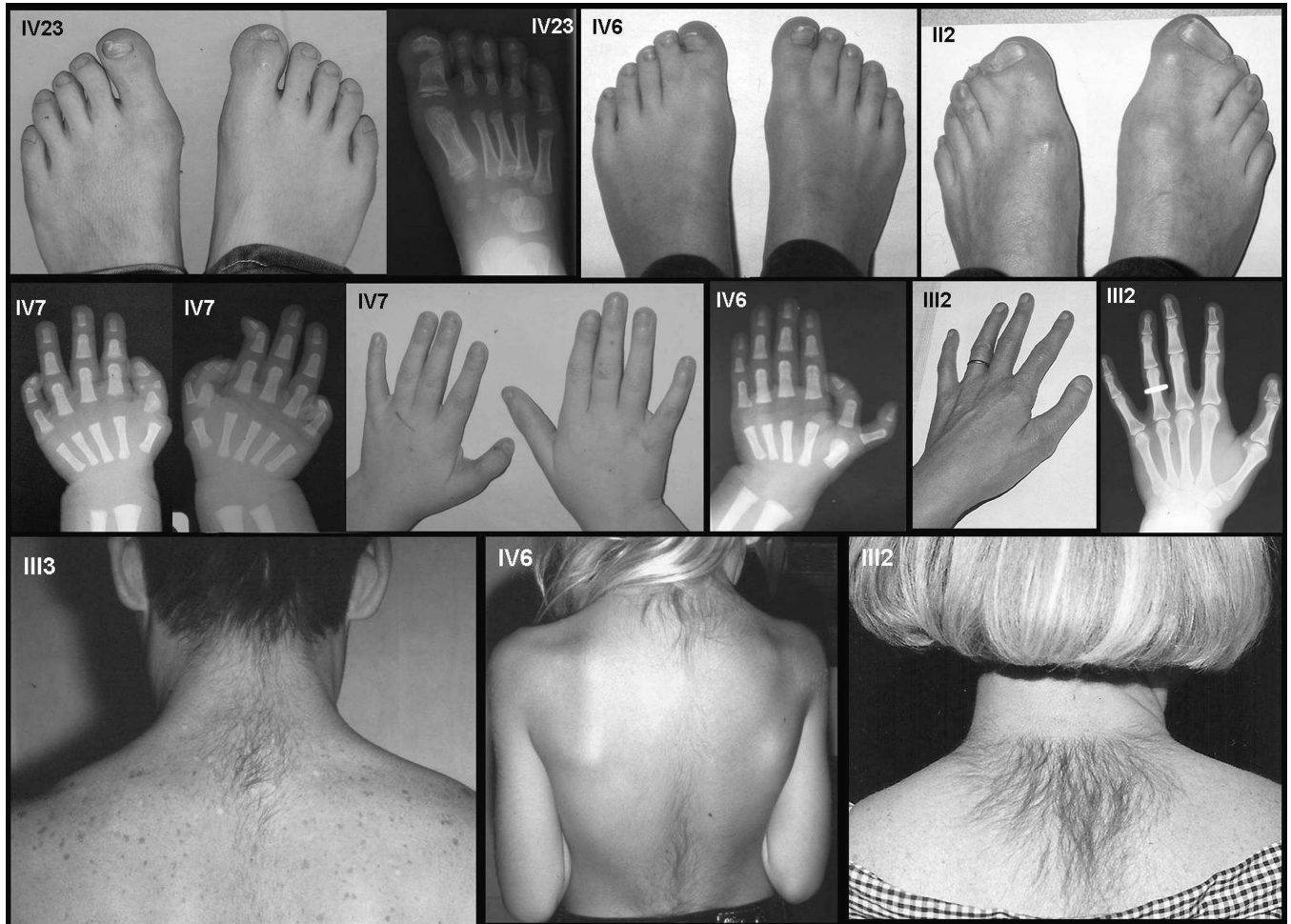


Figure 2 Clinical and radiological findings in patients affected with preaxial polydactyly and hypertrichosis. (a) Hand malformations are variable, ranging from triphalangeal thumb, biphalangeal partly duplicated thumb, preaxial extra ray to syndactyly between digits I and II. (b) Foot malformations are variable, ranging from large to duplicated hallux, potentially associated with syndactyly between the first and second rays. (c) Pictures showing upper back hypertrichosis, starting as a low posterior hairline and spreading down to the middle of the back. More clinical pictures are available in Supplementary Figures 1–3.

contains an element capable of repressing the transcriptional activity of the *SHH* promoter in *cis*, consistent with a silencer activity.

DISCUSSION

We report a large family affected with PPD and upper back hypertrichosis, with autosomal dominant inheritance and variable expressivity. To our knowledge, this phenotypic association has never been described before. The main differential diagnoses are non-syndromic PPDs (Supplementary Table 4), as the hypertrichosis of the back can be overlooked, especially in males. Non-syndromic PPDs are caused by gain-of-function mutations or copy number gains of the ZRS, a limb-specific enhancer of *SHH*. Besides, some cases of non-syndromic PPDs have been reported in association with *GLI3* mutations (OMIM#165240).^{15,16} *GLI3* is responsible for Greig cephalopolysyndactyly syndrome (GCPS, OMIM#175700) and Pallister–Hall Syndrome (PHS, OMIM#146510). GCPS can be very variable but usually presents with crossed polysyndactyly (PPD of lower limbs and postaxial polydactyly of upper limbs), macrocephaly, corpus callosum agenesis and sometimes craniosynostosis. In PHS, the polydactyly is usually meso-axial or postaxial and is associated with hypothalamic hamartoma. It is debated if *GLI3* could effectively be responsible for

non-syndromic polydactyly or if this would only be the milder end of GCPS or PHS spectrum.¹⁷ Hypertrichosis has not been reported in *GLI3*-associated syndromes, except for some cases harboring 7p13 microdeletions involving *GLI3*.¹⁸ This feature is therefore likely due to the contiguous gene deletion.

Given the central role of *SHH* in the anteroposterior polarization of the limb and in the follicle morphogenesis,^{10–12} we hypothesized that the PPD-hypertrichosis phenotype is consistent with a deregulation of *SHH* expression. No pathogenic variant or copy number variation was identified in the ZRS, which is the only limb-specific enhancer of *SHH* known to date. Nevertheless, the regulatory architecture of *SHH* necessary for limb development seems incompletely defined so far. Other limb-specific cis-regulatory elements of *SHH* are likely, as numerous PPD families with linkage at 7q36 do not present with ZRS alteration.^{8,9} The identification of a 4–6 kb deletion between ZRS and *SHH* in acheiropodia, which phenotype is consistent with the loss of *SHH* expression in the developing limb, is a further clue.

In this PPD-hypertrichosis family, linkage at the 7q36 locus upstream of the *SHH* gene was confirmed by SNP-arrays haplotyping. We identified the loss of a 2026 bp region in the gene desert 240 kb from the *SHH* promoter in our patients. This deleted region is

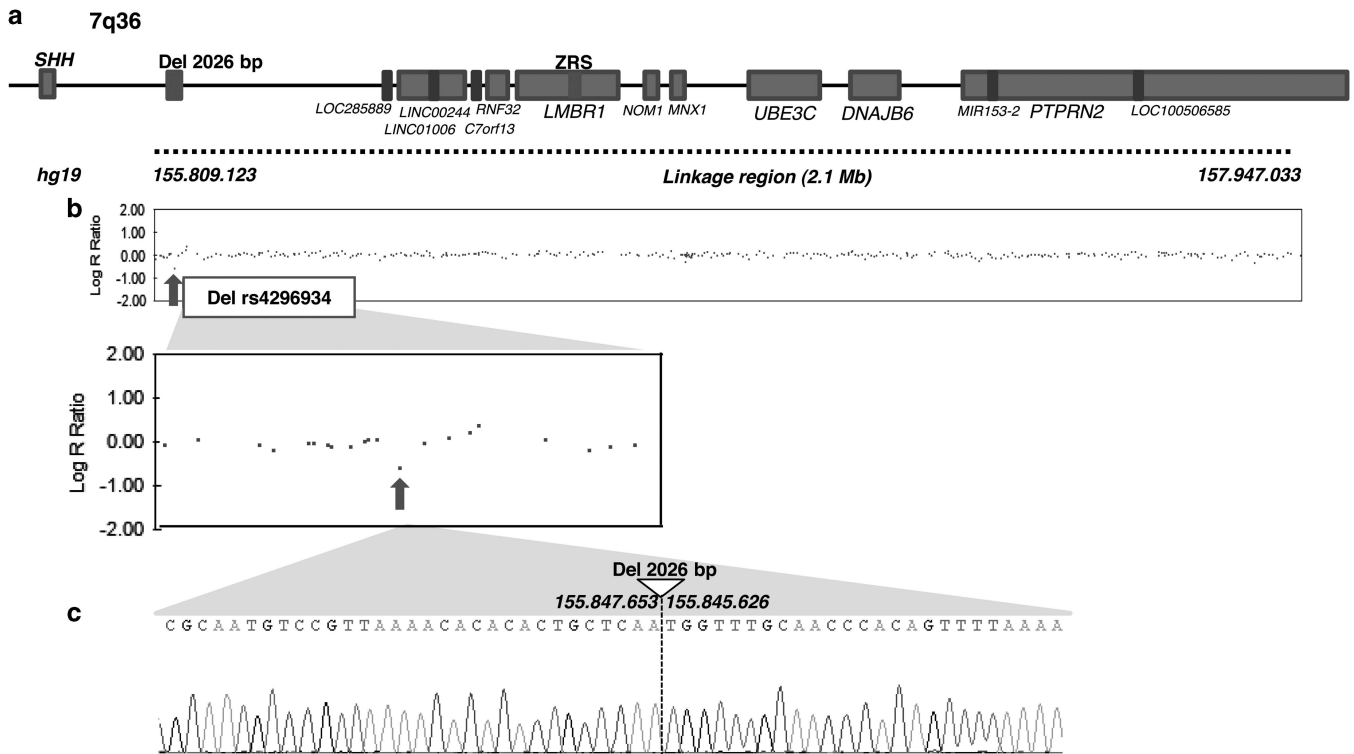


Figure 3 (a) Locus for PPD-hypertrichosis identified by SNP-arrays haplotyping (delimited by the dotted line). The region spans 2.1 Mb at 7q36 (chr7.hg19: g.155809123_157947033). It is located upstream of the *SHH* gene and encompasses the ZRS. (b) SNP array profile for the linkage region showing the heterozygous deletion of rs4296934 (arrow). (c) Sanger sequencing of the deletion breakpoint. Identification of a heterozygous 2026 bp deletion in the gene desert upstream *SHH* segregating in affected individuals.

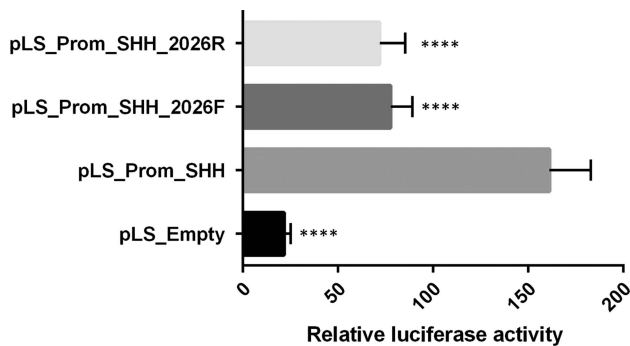


Figure 4 Functional study of the 2026 bp element. Relative luciferase expression was examined in Caco2 cells using a reporter construct pLS_Prom_SHH_2026 containing the 2026 bp sequence that is deleted in patients affected with PPD-hypertrichosis, in forward (F) or reverse (R) orientation. Reporter activity was normalized to the pLS_Prom_SHH control vector that do not contains any regulatory element upstream of the *SHH* promoter. In presence of the 2026 bp sequence and in absence of the promoter, the reporter activity is significantly reduced (**** $P < 0.0001$). Error bars represent the SD obtained from three independent experiments performed in triplicates.

partially conserved among species and absent from the Database of Genomic Variants and in 100 controls tested. Transfection experiments showed that the deleted sequence contains an element capable of repressing the transcriptional activity of the *SHH* promoter, consistent with a silencer activity. Therefore, we hypothesize that the deletion of this novel cis-regulatory element in our patients could explain *SHH* overexpression during limb development, leading to

PPD. However, our present data do not allow us to rule out that another molecular mechanism could be responsible for the phenotype: this deletion may lead to disrupted interactions between *SHH* and other regulatory elements; alternatively, a variant in a potential unknown regulatory element within the candidate region may remain undetected. Furthermore, *in vitro* studies of the regulation of genes involved in limb development are difficult to design because of the lack of relevant cell lines available. For these transfection experiments, we chose to use the human colon adenocarcinoma cell line Caco2 because it expresses *SHH* and has been used before successfully to study the limb-specific ZRS enhancer.¹⁹ The existence of multiple regulatory elements in addition to the ZRS is likely, similarly to the regulatory clusters orchestrating the spatiotemporal pattern of *SHH* expression necessary to the development of the central nervous system^{20,21} and the epithelial linings.²² The regulatory element identified may interact with the *SHH* promoter located at long distance by formation of a chromosome loop, similarly to the ZRS enhancer.²³ The *SHH* spatial and temporal expression pattern may be directed by the assemblage of these two (or more) autonomous regulatory activities.

In silico analysis of the candidate sequence shows numerous predicted binding sites for transcription factors. Among these, four transcription factors are good candidates to explain the regulatory activity of our sequence, given the literature data. ETS and HAND transcription factor families have, respectively, six and four predicted binding sites on the sequence that is deleted in the PPD-hypertrichosis family. These factors, in particular ETS1/ETV4/ETV5 (OMIM#164720, 600711, 601600) and HAND2 (OMIM#602407), are known to have central roles in the *SHH* spatial pattern during limb development via

their direct binding to the ZRS.^{24,25} Furthermore, variants of ETV4/5 binding sites on the ZRS are responsible for its ectopic activity leading to PPD.²⁵ To our knowledge, these transcription factor families have not been involved in follicle morphogenesis or growth.

Binding sites for GATA and NKX family factors are over-represented on the 2 kb sequence and especially along its more evolutionary conserved part, with, respectively, 16 and 33 predicted sites, suggesting a 'homotypic clustering'. The latter, corresponding to the enrichment of multiple binding sites for the same transcription factor, is a common feature of cis-regulatory elements in vertebrate and invertebrate.²⁶ These two families of transcription factors are known to act synergistically to regulate tissue-specific gene expression during development.²⁷ This co-regulation activity has been well described for GATA4-NKX2.5 in cardiogenesis^{28–30} and for GATA6-NKX2.1 in lung development.^{27,31} To our knowledge, no major role in limb development has been described for NKX factors so far. Interestingly, it has recently been demonstrated that *Gata4* and *Gata6* are differentially expressed in the anterior mesenchyme of the developing limb buds in mice.³² *Gata6* represses ectopic expression of *Shh* in the anterior region of the limb bud by directly binding to the ZRS. The limb bud-specific *Gata6* deletion results in ectopic expression of *Shh* and its target genes in the anterior margin, therefore resulting in PPD. A simultaneous deletion of *Gata6* and *Shh* rescues the phenotype. Conversely, forced-expression of *Gata6* throughout the limb bud downregulates *Shh*, resulting in a decreased number of digits. Therefore, Gata transcription factors are crucial negative regulators of ectopic *Shh* expression during limb development. Furthermore, these factors contribute to transcriptional regulation by facilitating chromosome looping, thereby mediating long-range gene regulation.³³ Given these observations, we hypothesize that GATA transcription factors, and in particular GATA6, could bind to the regulatory element deleted in the PPD-hypertrichosis patients, conferring its silencing activity.

Given the role of SHH during follicle morphogenesis and growth, it is likely that its deregulation is also responsible for the hypertrichosis in the family. Wnt/ β -catenin signaling relayed through *Shh* and *Bmp* signals is the principal regulatory mechanism of hair follicle development.³⁴ Studies in mice showed that follicle morphogenesis and hair growth is altered when *Shh* is either downregulated^{10–12} or overexpressed.³⁵ This pathway is deregulated by knock-out of *Gata3* in mice, the animals showing abnormal hair formation.³⁶ Therefore, GATA transcription factors are candidates for interacting with the regulatory element identified, responsible for deregulation of *SHH* in the developing limb and hair follicle.

Chromatin immunoprecipitation experiments may be conducted to identify which transcription factors are involved in the regulatory activity of the 2 kb element described. Similarly to the ZRS, the interaction between this cis-regulatory element and the *SHH* promoter located at a 240 kb distance could occur by chromosomal looping. Chromosome conformation capture experiments may be performed to validate this hypothesis. Unfortunately, *in vivo* experiments would be difficult to design for two reasons: first, the limited choice of a relevant animal model for the two phenotypes (ie, polydactyly and hypertrichosis); second, the weak inter-species conservation of the potential regulatory element restricting even more this choice. Consequently, study of this newly suspected *SHH* limb regulatory element in other families affected with PPD-hypertrichosis may be the best approach to confirm our findings. Nevertheless, this phenotype has never been described before and is therefore presumed to be an extremely rare condition in humans. Of note, screening of the 2 kb element by sequencing and quantitative PCR of 20 patients affected

with isolated PPD (for whom ZRS sequence and copy number variation have been previously ruled out) revealed no molecular variation (data not shown).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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