

ARTICLE

Mutation analysis of a large Chinese pedigree with congenital preaxial polydactyly

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Mutations in the long-range limb-specific *cis*-regulator (ZRS) could cause ectopic *shh* gene expression and are responsible for preaxial polydactyly (PPD). In this study, we analyzed a large Chinese isolated autosomal dominant PPD pedigree. By fine mapping and haplotype construction, we located the linked region to a 1.7 cM interval between flanking markers D7S2465 and D7S2423 of chromosome 7q36. We directly sequenced the candidate loci in this linked region, including the coding regions of the five genes (*HLXB9*, *LMBR1*, *NOM1*, *RNF32* and *C7orf13*), the regulatory element (ZRS) of *shh*, the whole intron 5 of *LMBR1* which contained the ZRS, and 18 conserved noncoding sequences (CNSs). Interestingly, no pathogenic mutation was identified. By using real-time quantitative PCR (qPCR), we also excluded the ZRS duplication in this pedigree. Our results indicate that, at least, it is not the mutation in a functional gene, CNS region or duplication of ZRS that cause the phenotype of this pedigree. The etiology of this PPD family still remains unclear and the question whether another limb-specific regulatory element of *shh* gene exists in the noncoding region in this 1.7 cM interval remains open for future research.

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Introduction

Vertebrate limb development malformations could lead to both polydactyly (extra digits) and oligodactyly (too few digits).¹ Polydactyly is the most frequently observed congenital malformation with incidence rate between 5 and 19 per 10 000 live births.² Polydactyly can occur

either as an isolated disorder or syndrome. Furthermore, polydactyly can be classified into preaxial polydactyly (PPD) and postaxial polydactyly (PAP) according to its anatomical location. PPD phenotype is highly variable within families and ranges from a simple addition of a phalanx in triphalangeal thumb to whole digit duplication and tibial aplasia. According to Temtamy and McKusick,³ PPD can be divided into four types: type I (PPD-I) (OMIM 174400) is the duplication of one or more of the skeletal components of a biphalangeal thumb; type II (PPD-II) (OMIM 174500) is the polydactyly of a triphalangeal thumb; type III (PPD-III) (OMIM 174600) is the polydactyly of an index finger; type IV (PPD-IV) (OMIM 174700) is the polysyndactyly.

In the developing limb, asymmetric expression of Sonic Hedgehog (*shh*) in the zone of polarizing activity (ZPA) in posterior mesoderm is required for proper

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anterior/posterior (A/P) limb patterning.⁴ But in animal models and PPD families, mutations do not alter the *shh* coding sequence, which suggested that abnormal ectopic expression of *shh* is sufficient to induce supernumerary digits.^{5–7} Several unrelated PPD families under early genetic analysis had refined the PPD locus to a 450 kb region on chromosome 7q36, which covered five genes: *HLXB9*, *LMBR1*, *NOM1*, *RNF32* and *C7orf13*.^{8–13} So far, all described PPD pedigrees were linked to this locus.^{8–17} Moreover, other limb disorders, such as acheiropodia¹⁸ (OMIM 200500), complex polysyndactyly,¹⁴ acropectoral syndrome¹⁹ (OMIM 605967) and currarino syndrome²⁰ (OMIM 176450), were also mapped to this locus, which demonstrated that gene or regulatory elements vital for limb development are located in this region. Subsequent study of a spontaneous balanced translocation breakpoint in a girl with PDD and a transgenic inserted *Sq* mouse indicated that intron 5 of *LMBR1* contained a *shh* limb-specific regulatory element.²¹ Subsequent comparative genomic analysis revealed that this *shh* regulatory element (ZRS) was conserved in tetrapod lineage and teleost fishes but was absent or deleted among limbless reptiles and amphibians.²² In recent years, different point mutations in ZRS have been reported in PPD/TPTPS/SD4 families and in mouse and cat mutants.^{9,13,22–25}

To investigate the etiology of a large isolated PPD pedigree, which is the first Chinese PPD-II/III pedigree for molecular study, we performed linkage analysis on this pedigree. By using a fine mapping and haplotype analysis, we located the affected region to 1.7 cM between markers D7S2465 and D7S2423, including the 450-kb region mentioned above. But mutation screen by DNA sequencing gave us an unexpected result: no pathogenic mutation was found in the reported ZRS of *shh*, and subsequent extended screen indicated that there was no pathogenic mutation in the adjacent coding genes and conserved noncoding sequences (CNSs) in this linked 1.7 cM interval. As duplication involving ZRS is reported to be new pathogenic mechanism underlying TPTPS and SD4,^{16,17} we also performed quantitative PCR (qPCR) in this pedigree. Our result showed that such rearrangement was not present in this family. Up to this stage the unconserved regions in this interval have not been determined and the question whether there is another regulatory element in the unconserved noncoding regions of this linked 1.7 cM interval remains open for future research.

Subjects and methods

Subjects

In this study, we recruited a six-generation pedigree including 45 Chinese Han individuals (21 affected members and 24 normal relatives) from a small village in Zhejiang Province (Figure 1). Meanwhile, 20 normal healthy individuals were recruited as controls. The detailed

clinical investigation was performed for all affected members and peripheral blood samples were obtained for DNA analysis. Written informed consent was obtained from every subject. Autosomal-dominant inheritance of the phenotype was ascertained by the presence of affected individuals in each of the six generations and with a male–male transmission. The phenotype showed a complete penetrance in this pedigree.

Clinical phenotype

Total of 45 subjects were collected in this study, including 21 affected members, 12 healthy partners and 12 unaffected sibs. The affected members all have bilateral PPD and show hexadactyly of hands and feet (Figure 2). On the basis of physical examination, patients have the duplication of two phalanges or three phalanges thumb and show the phenotype of opposable thumb or non-opposable thumb. All affected members present a strikingly similar phenotype on feet with six fully developed toes. The supernumerary ray is localized between the first and the second ray. On the X-ray, the extra toe is biphalangal and has the appearance of a somewhat hypoplastic (duplicated) hallux (Figure 2b). No other abnormal of hand or feet and associated systemic anomalies are observed in this family. Therefore, we classified the clinical character of the family as isolated PPD-II/III according to Temtamy and McKusick's classification.³

Genotyping

The genomic DNA was extracted from peripheral blood by standard phenol/chloroform method. Nine microsatellite markers: D7S550, D7S3037, D7S2465, CGR13, CGR12, HING1, D7S559, CGR16 and D7S2423 were genotyped. The primers information was obtained from the Genome Database (GDB), the Génethon and the Cooperative Human Linkage Center collection (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome>; <http://www.genethon.fr/>; <http://www.chlc.org/>) and the report from Heus *et al.*¹² PCR was performed in a 15 μ l volume with 10 ng genomic DNA as template, 1.5 μ l PCR 10 \times buffer, 1.5 μ l dNTP mix (2.5 mM), 1 μ l primers, 1 μ l MgCl₂ (15 mM), 0.12 U of Taq DNA polymerase (Takara, Dalian, China) and 9 μ l DW water. Thermal cycling in a Gene Amp 9700 (PerkinElmer, Waltham, MA, USA) thermal cycler was performed at 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a 10 min extension at 72 °C and a hold at 10 °C. Then the PCR products were denatured and size fractionated on 5% denatured polyacrylamide gel (PAGE) run on ABI 377 sequencer. GS350 size standard was used as internal standard run in the same lane with the markers. Alleles read and scored by use of Genescan and Genotyper 2.0 software (ABI PerkinElmer, Waltham, MA, USA) were confirmed by eye.

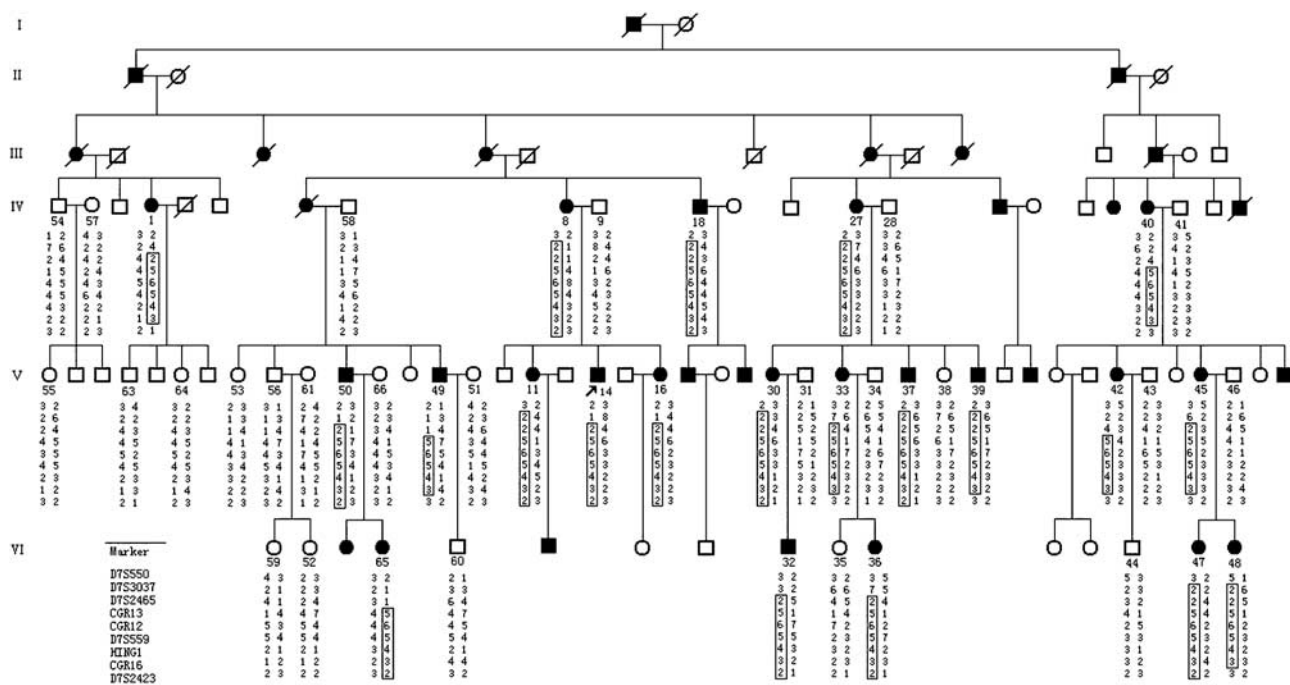


Figure 1 Pedigree of the family studied and haplotypes obtained with 9 microsatellite DNA markers on chromosome 7q36. Solid symbols represent affected individuals; open symbols represent unaffected individuals. The sequence of markers is from centromere to telomere. The affected haplotype is shown in the rectangles.

Linkage analysis and haplotype construction

Two-point LOD scores were calculated by using MLINK program of Linkage software package (version 5.2). Linkage was determined in an autosomal-dominant model under penetrance of 95%. The gene frequency of disease was set to 0.001 and the mutation rate was set to zero. The recombination frequencies between male and female were assumed equal. Allele frequencies for all markers were kept equal. Haplotypes were constructed with software Simwalk2 (www.genetics.ucla.edu).

Sequence analysis of candidate regions

The candidate regions were amplified with genomic DNA. The conditions of PCR were identical to those mentioned above. PCR products were purified on spin columns (Watson Biotechnologies Inc., Shanghai, China) and were directly sequenced for both strands using BigDye Terminator Cycle Sequence Kit 3.1 (ABI Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. The sequencing results were processed by DNASTAR software (DNASTAR) and manual rectification.

Real-time quantitative PCR

qPCR was performed on ABI Prism 7000 Sequence Detection System in a total volume of 20 μ l in each well containing 10 μ l of SYBR Premix Ex Taq (Takara), 1 μ l of

genomic DNA (20 ng), and 5 μ l of Primers (400 nM each) with five replicates per sample. PCR conditions followed the manufacturer's protocol with 95 $^{\circ}$ C for 10 min and 40 cycles of 95 $^{\circ}$ C 15 s/60 $^{\circ}$ C 1 min. By using calibrator samples of normal control genomic DNA, the quantification of the target genes was normalized to an assay from chromosome 21 and the relative copy number was determined based on the $\Delta\Delta C_t$ method.²⁶ The ZRS was detected with primers previously described.¹⁶ The experiments were repeated five times and a conservative \log_2 ratio threshold of 0.3 and -0.3 was used for copy number gains or losses.

Results

Linkage analysis

A maximum two-point LOD score (Z_{max}) of 7.822 was obtained at recombination fraction (θ) 0.00 for marker HING1 within the *LMBR1* gene (Table 1), which implied strong linkage between PPD phenotype of this kindred and chromosome 7q36. Haplotype construction and recombination analysis narrowed the candidate region to a 1.7 cM region between D7S2465 and D7S2423, which covered the previous reported 450-kb interval (Figure 1).¹² Five genes (*HLXB9*, *LMBR1*, *NOM1*, *RNF32* and *C7orf13*), as well as the regulatory element ZRS within the intron 5 of *LMBR1*, were located inside this 1.7 cM interval.⁸⁻¹³

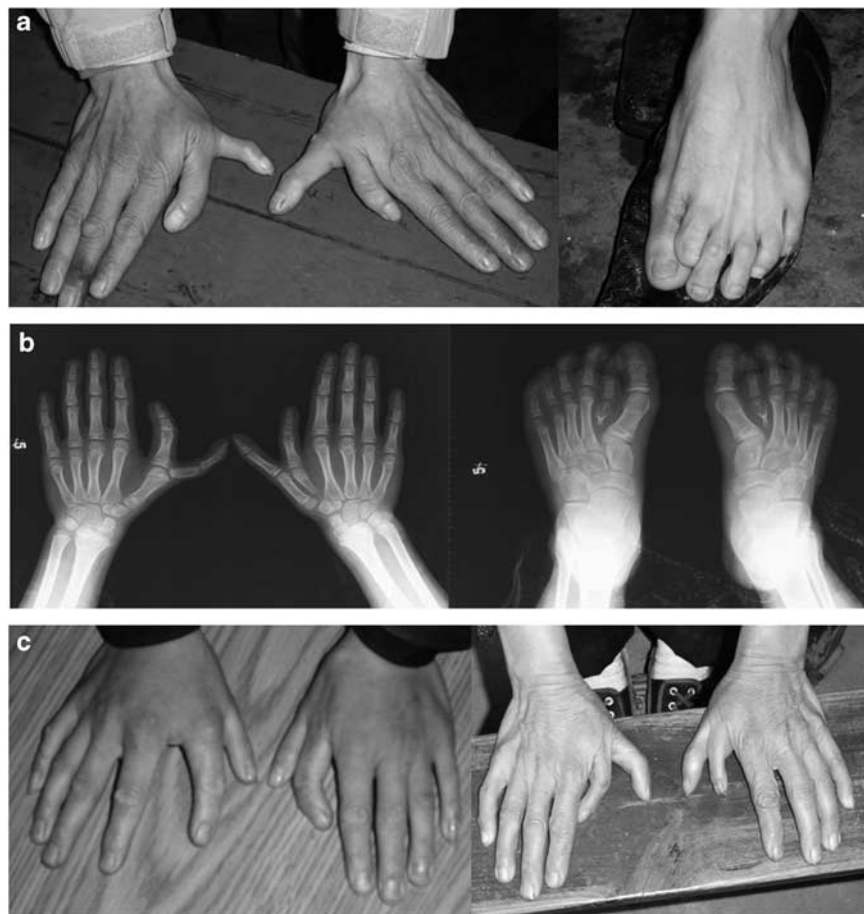


Figure 2 Clinical phenotypes of affected individuals. (a) Normal images of hands and feet of affected V-37, which showed the hexadactyly. (b) The radiograms image of VI-47 shows a bilateral duplication of triphalangeal thumbs and the biphalangal great toes. (c) Normal images of affected V-39 and V-11 showed the opposable thumb and non-opposable thumb.

Mutation screen of candidate genes

We performed a comprehensive mutation screening in the coding regions of *HLXB9*, *LMBR1*, *NOM1*, *RNF32* and *C7orf13*. No sequence alterations of the amino acid coding of these five genes were found. Then the 3'-untranslated regions and their intron–exon boundaries of the five genes, as well as the *cis*-acting regulator of *shh* (ZRS), were also screened and no pathogenic mutation was found. In addition, no deleterious sequence changes were found in all the alternative splicing transcripts of the five genes and three annotated genes (*Loc100129517*, *Loc100129551* and *Loc645249*) in the region.

Then, the whole intron 5 of *LMBR1* containing the ZRS was sequenced. A total of 18 single-nucleotide polymorphisms (SNPs) were found (Table 2) but none of them was pathogenic mutation. Furthermore, all of them were also detected in normal healthy controls. These results showed that there is no deleterious mutation in the coding regions and reported regulatory element (ZRS) of this 1.7 cM interval in this pedigree.

As reported in families of acheiropody, a severe disorder presenting with bilateral amputations of upper and lower extremities, a homozygous deletion of 4–6 kb containing *LMBR1* exon 4 was the etiology. We also scanned the ~7 kb region of acheiropody deletion in our pedigree, but still no pathogenic mutation was identified.

Identification and sequencing of the conserved noncoding sequence

Highly CNSs among diverse species have been predicted to be involved in regulation of transcription, DNA replication, chromosome pairing and chromosome condensation.²⁷ Recently, CNS was also suggested to be associated with vertebrate development²⁸ and in fact, the ZRS of *shh* was highly conserved in tetrapod lineage and even in teleost fishes.²² So we sequenced CNSs within this 1.7 cM interval.

The linked 1.7 cM region of human and its homologous sequences from several widely divergent organisms (mouse, rat, dog and chick) were downloaded

Table 1 Two-point LOD scores between PPDII/III and markers on chromosome 7q36

Position	Marker	Recombination fraction						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
155 210 550	D7S550	-6.640	-2.816	-0.834	-0.073	0.404	0.409	0.233
155 446 337	D7S3037	$-\infty$	-8.524	-3.872	-2.067	-0.627	-0.115	0.024
155 807 517	D7S2465	-9.826	0.389	2.267	2.772	2.661	1.998	1.067
156 035 959	CGR13	6.683	6.564	6.076	5.439	4.094	2.694	1.288
156 101 549	CGR12	6.604	6.486	6.002	5.376	4.061	2.679	1.292
156 165 754	D7S559	5.442	5.571	5.543	5.171	4.072	2.749	1.326
156 210 334	HING1	7.822	7.686	7.132	6.415	4.904	3.300	1.623
156 612 825	CGR16	6.664	6.536	6.016	5.346	3.942	2.471	1.017
157 290 784	D7S2423	-5.639	-4.558	-2.382	-1.451	-0.638	-0.263	-0.074

Table 2 Information of the 18 SNPs in intron 5 of *C7orf2*

NCBI rs number	Alleles	Position on B36
rs17837692	C/T	156 250 504
	C/T	156 251 590
	C/G	156 256 601
	G/A	156 258 291
rs10266196	G/C	156 262 271
	T/G	156 266 345
rs11770562	C/G	156 267 720
rs10281201	C/T	156 270 821
	A/C	156 271 700
rs6969301	C/T	156 271 824
	T/G	156 273 398
	A/G	156 274 634
rs10949603	C/T	156 274 801
rs10254391	C/G	156 277 328
rs10229091	C/T	156 277 665
rs6949624	C/G	156 277 945
rs12535434	C/T	156 278 854
rs1017137	A/G	156 279 436

Table 3 The locations of 18 CNS on human chromosome 7q36

CNS	Position in human chromosome 7
CNS1	156 043 780–156 043 923
CNS2	156 046 845–156 047 037
CNS3	156 053 768–156 054 505
CNS4	156 087 968–156 088 474
CNS5	156 100 086–156 100 681
CNS6	156 152 259–156 152 803
CNS7	156 207 906–156 208 298
CNS8	156 210 391–156 210 889
CNS9	156 210 981–156 211 218
CNS10	156 232 373–156 232 774
CNS11	156 249 495–156 249 625
CNS12	156 265 133–156 265 330
CNS13	156 274 992–156 275 037
CNS14	156 276 556–156 277 336
CNS15	156 278 065–156 278 252
CNS16	156 485 655–156 485 855
CNS17	156 488 554–156 488 670
CNS18	156 490 365–156 490 506

Abbreviation: CNS, conserved noncoding sequence.

from UCSC (<http://genome.ucsc.edu/>; human (chromosome7: 156 033 000–156 493 000 Mar 2006, UCSC hg18); mouse (July 2007, UCSC mm9); rat (Nov 2004, UCSC rn4); dog (May 2005); chicken (Feb 2004, UCSC version gal2)). The multiple species sequences were aligned by VISTA (<http://www-gsd.lbl.gov/vista>) and displayed using PileUP (The GCG Wisconsin Package at the HGMP, Hinxton, UK).²⁹ All sequences were soft-masked and aligned using SLAGAN alignment tool kit (<http://www-gsd.lbl.gov/vista>).

A total of 18 CNSs shared by human–mouse, human–rat, human–dog and human–chicken alignment were found and subsequently sequenced (Table 3). After sequencing analysis, we detected 10 SNPs among the 18 CNSs and all the 10 SNPs were also present in normal healthy controls. Therefore, they could not be pathogenic mutations accounting for PPD.

Identification of the ZRS duplication

ZRS duplication was found in some families of TPTPS and SD4 phenotype.^{16,17} qPCR was used to detect whether the

copy number changes of ZRS was present in this PPD pedigree. We examined 8 affected individuals, 8 normal individuals from the family and 9 unrelated healthy Han Chinese controls with the same ethnic background. The duplication of ZRS was not detected and the mean relative copy number was 1.0 (mean value 0.95–1.08) in the affected group.

Discussion

It is not easy to choose proper organisms for interspecies sequence comparison of a particular DNA region due to the inconsistent mutation rates across the genome,³⁰ which might be one of the factors contributing to our negative result. Another limitation for multispecies sequence comparison may overlook the regulatory element whose

sequence or position in the genomes has not been conserved across phyla, such as some enhancers.³¹

In previous PPD studies, there are only 13 pedigrees in which the responsible point mutations were identified.^{13,16,17,23,25} On the other hand, pathogenic mutations are still remained unidentified in most PPD families.^{8,11–13,23} Furthermore, although the 7 pathogenic point mutations identified in above 13 pedigrees were all located in the ZRS of *shh*, these mutation sites were scattered in the ZRS and no clustering of mutations toward domain-like structure was evident. In fact, no known regulatory domain had been defined by bioinformatics method, so far.¹³ These information suggested that the network of *shh* regulation was much complex and is poorly understood.

Recently, the copy number variations, such as duplication, has been shown to be the underlying cause for human genetic disorders.³² As *shh* gene is expressed in different tissues and developmental stages, more accurate and multiple complex regulators are necessary. Very recently, two different research teams have found a genomic duplication fragment containing ZRS in TPTPS and SD4 families,^{16,17} but the precise molecular mechanism about how the duplication cause human limb disorders is unclear. The phenotype of TPTPS and SD4 are more severe and complicated than isolated PPD, which cannot be simply explained by *shh* ectopic expression, though all the disorders are confined to human limbs. Duplication of ZRS may have more severe deleterious effect on *shh* expression than single-nucleotide alterations in the ZRS. As all affected individuals in our PPD kindred only had the simple phenotype of bilateral hexadactyly hand and foot without any other features, the presumed mutation in this PPD family should be one causing the disruption of distal A/P axis formation and mildly dysregulate *shh* expression. qPCR was used to detect the duplication of ZRS and showed that ZRS rearrangement was not the pathogenic mutation in our pedigree, which was consistent with this postulation based on the phenotype.

The question whether there is other regulatory element of *shh* in the defined 1.7 cM interval, in addition to the known ZRS, is intriguing. The studies of *de novo* reciprocal t(5,7)(q11,136) translocation and the acheiropody family with 4–6 kb deletion containing *LMBR1* exon 4 implied the presence of other limb-specific regulator of *shh* expression.^{18,21} In our pedigree, the fact that all the coding regions and conserved regions were excluded from containing pathogenic mutation suggests that the other regulatory element could, if it exists, locate in unconserved regions. It seems likely that the only way to detect the proposed pathogenic mutation for our pedigree is to thoroughly sequence the remained unconserved regions in this 1.7 cM interval. The eventual characterization of the pathogenic mutation for this family could help better understand the complex developmental gene regulatory circuitry in human limb embryogenesis.

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