Am. J. Hum. Genet. 63:711–716, 1998

Mutations in Fibroblast Growth-Factor Receptor 3 in Sporadic Cases of Achondroplasia Occur Exclusively on the Paternally Derived Chromosome

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Summary

More than 97% of achondroplasia cases are caused by one of two mutations (G1138A and G1138C) in the fibroblast growth factor receptor 3 (FGFR3) gene, which results in a specific amino acid substitution, G380R. Sporadic cases of achondroplasia have been associated with advanced paternal age, suggesting that these mutations occur preferentially during spermatogenesis. We have determined the parental origin of the achondroplasia mutation in 40 sporadic cases. Three distinct 1-bp polymorphisms were identified in the FGFR3 gene, within close proximity to the achondroplasia mutation site. Ninety-nine families, each with a sporadic case of achondroplasia in a child, were analyzed in this study. In this population, the achondroplasia mutation occurred on the paternal chromosome in all 40 cases in which parental origin was unambiguous. This observation is consistent with the clinical observation of advanced paternal age resulting in new cases of achondroplasia and suggests that factors influencing DNA replication or repair during spermatogenesis, but not during oogenesis, may predispose to the occurrence of the G1138 FGFR3 mutations.

Introduction

Achondroplasia (MIM 100800) is the most common form of dwarfism in humans. The disorder is inherited in an autosomal dominant manner, with full penetrance. Two mutations, G1138A and G1138C, in exon 10 of the FGFR3 gene result in a specific amino acid substitution (G380R) (Rousseau et al. 1994; Shiang et al. 1994) and account for more than 97% of all reported cases (Bellus et al. 1995*a*). On the basis of the incidence of achondroplasia, FGFR3 nucleotide 1138 is among the most highly mutable single nucleotides known in the human genome. Specific FGFR3 mutations also have been identified in disorders related to achondroplasia, including thanatophoric dysplasia types I and II (Tavormina et al. 1995; Rousseau et al. 1996), hypochondroplasia (Bellus et al. 1995*b*), and severe achondroplasia with developmental delay and acanthosis nigricans dysplasia, a recently described skeletal dysplasia (Francomano et al. 1996; G. A. Bellus, M. J. Bamshad, and C. A. Francomano, unpublished data). In addition, FGFR3 mutations have been identified in several craniofacial disorders, including nonsyndromic craniosynostosis and Crouzon syndrome with acanthosis nigricans (Meyers et al. 1995; Bellus et al. 1996).

Sporadic cases of achondroplasia and other dominant genetic disorders have been associated with advanced paternal age, suggesting that these mutations occur preferentially during spermatogenesis (Penrose 1957; Thompson et al. 1986). Using single-base-pair polymorphisms in the FGFR3 gene that are proximate to the site of the achondroplasia mutation, we have determined the parental origin of the achondroplasia mutation in 40 sporadic cases. Heteroduplex analysis was used to screen for FGFR3 intron polymorphisms, identifying polymorphisms in FGFR3 introns 9 and 10. DNA from 99 families with sporadic occurrences of achondroplasia was analyzed for heterozygosity of these polymorphisms, with informative probands heterozygous for at least one of the polymorphisms. The amplification-refractory mutation system (ARMS) (Moloney et al. 1996) or sequencing was used to determine the phase of the polymorphism, together with the achondroplasia mutation or normal allele, in the probands. Determination

Received December 10, 1997; accepted for publication July 15, 1998; electronically published August 7, 1998.

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0002-9297/98/6303-0009$02.00

Table 1

FGFR3 Oligonucleotide Primer Sequences

Primer[a]	Location	Sequence
CD5-F	Exon 9	5′-GGTTTTCTCATCACTCTGCG-3′
1138wt-R	Exon 10	5′-GAACAGGAAGAAGCCCACCGC-3′
1138mut-R	Exon 10	5′-GAACAGGAAGAAGCCCACCGT-3′
1138wt-F	Exon 10	5′-ATGCAGGCATCCTCAGCTAGG-3′
1138mut-F	Exon 10	5′-ATGCAGGCATCCTCAGCTAGA-3′
int10-R	Exon 11	5′-GGTGTGTTGGAGCTCATGG-3′
int10-F	Exon 10	5′-CCACCGTGCACAAAGATCTC-3′
961-F	Exon 9	5′-CTGCCTGGCGGGCAATTCTA-3′
DT-R	Exon 10	5′-GGAGATCTTGTGCACGGTGG-3′
DT-F	Exon 10	5′-AGGAGCTGGTGGAGGCTGA-3′
1395-R	Exon 11	5′-GCTCGAGCTCGGAGACATTG-3′
I9A-F	Intron 9	5′-TCACTCCTGGCCCTGTGCC-3′
I9B-F	Intron 9	5′-GCGCGTGCTGAAGTTCTG-3′
I10B-R	Intron 10	5′-GTGAGCAGAGACGAGGAGAG-3′

[a] F = forward, R = reverse, wt = wild type, and mut = mutant.

of the polymorphic variant status in the parents allowed the identification of the parent from whom the mutant allele had been inherited. This analysis revealed that the achondroplasia mutation occurred on the paternal chromosome in all 40 informative probands. The paternal origin of the mutant achondroplasia allele suggests that factors influencing DNA replication or repair during spermatogenesis, but not during oogenesis, may predispose to the occurrence of achondroplasia.

Subjects and Methods

Subjects

Ninety-nine achondroplasia families were ascertained, with informed consent, by genetics clinics at various locations in the United States, England, France, Finland, and Poland. Each family consisted of one affected child, with both parents being of average stature. In 97 families, the proband had the G1138A achondroplasia mutation within FGFR3 exon 10, whereas the other 2 of the 99 probands were heterozygous for the G1138C mutation. As far as we know, none of the families are related to each other. Paternal and maternal ages at the time of conception or birth of the proband were available for 39 and 37 of the 40 informative families, respectively. The mean paternal and maternal ages in these families were, respectively, 35.58 ± 7.18 and 30.98 ± 4.21 years.

Identification of Polymorphic Variants

Since the recurrent achondroplasia mutation is in exon 10 of the FGFR3 gene, regions of the gene that are close to this site were examined for polymorphisms. Primer pairs (int10-F and int10-R, 961-F and DT-R, or DT-F and 1395-R) (table 1) were used to amplify FGFR3 intron 9 or FGFR3 intron 10. Polymorphisms were identified by either SSCP or heteroduplex analysis and/or by sequencing. Primers 961-F, I9A-F, and I9B-F were used as sequencing primers for intron 9, and primers 1395-R and I10B-R were used to sequence intron 10. Sequencing was performed either directly, by use of Dynabeads M-280 streptavidin (Dynal) (Thein and Hinton 1991), or by use of an ABI 377 automated DNA sequencer (Applied Biosystems).

Analysis of the Polymorphic Sites

Polymorphisms that altered a restriction site were analyzed by means of the ARMS method (Moloney et al. 1996). In brief, in the probands, allele-specific PCR reactions were designed to amplify an FGFR3 gene region that included both the site of the polymorphism and the site of the common achondroplasia mutation. Allele-specific PCR was performed on either the mutant or normal chromosome, by means of oligonucleotide primers specific for either the normal allele (1138 wt-F or 1138wt-R) or the achondroplasia allele (1138mut-F or 1138mut-R), and the appropriate corresponding primer (CD5-F or int10-R) (table 1). Allele-specific primers were designed to create a mismatch at the nucleotide adjacent to the 3′ nucleotide, with the 3′ nucleotide corresponding to FGFR3 nucleotide 1138 (Kwok et al. 1990) (fig. 1). The normal-allele PCR primers (1138wt-F and 1138wt-R) contained the wild-type nucleotide G (or C, on the

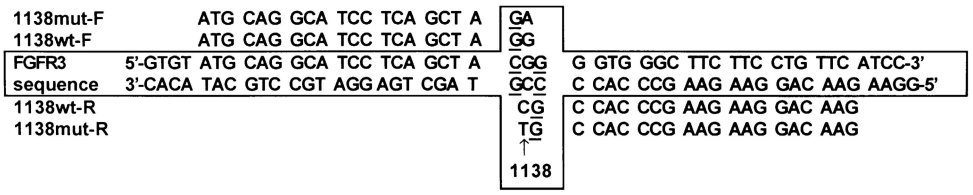

Figure 1 FGFR3 achondroplasia mutant and wild-type allele-specific oligonucleotide primers, designed to create a mismatch at the nucleotide adjacent to the 3′ nucleotide, with the 3′ nucleotide corresponding to FGFR3 nucleotide 1138, the base mutated in the common achondroplasia mutation analyzed in this study. The normal-allele PCR primers (1138wt-F and 1138wt-R) contained the wild-type nucleotide G (or C on the reverse primer) as the 3′ base, maximizing the mismatch with the mutant allele. The mutant-allele PCR primers (1138mut-F and 1138mut-R) contain the achondroplasia-mutation nucleotide A (or T on the reverse primer) as the 3′ base, maximizing the mismatch with the normal allele.

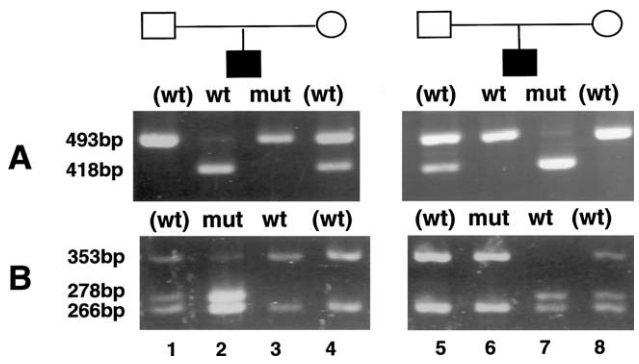

Figure 2 *Pml*I (*A*) and *Pfl*MI (*B*) restriction digestion of allele-specific PCR products, as recommended by the manufacturer. The sizes of products of restriction digestion are shown. Analyses of four families are presented. Allele-specific PCR of genomic DNA was performed with either a normal-sequence (wt) oligonucleotide primer or an oligonucleotide primer specific for the common achondroplasia mutation (mut) (see fig. 1). The allele-specific primer used in the PCR reactions is depicted as wt or mut. Reactions using DNA from the probands are shown in lanes 2, 3, 6, and 7. PCR using genomic DNA from the affected child was performed with either a wt oligonucleotide primer or a mut primer. Parental DNAs are shown in lanes 1, 4, 5, and 8. PCR of parental genomic DNA was performed with a wt primer. In each case shown, the allele amplifying with the mut primer is inherited from the father.

reverse primer) as the 3′ base, maximizing the mismatch to the mutant allele (fig. 1). The mutant-allele PCR primers (1138mut-F and 1138mut-R) contain the achondroplasia-mutation nucleotide A (or T, on the reverse primer) as the 3′ base, maximizing the mismatch to the normal allele (fig. 1). Digestion of the PCR product, with either *Pfl*MI or *Pml*I, distinguished the phase of the polymorphism and mutation. For the intron 9 *Pfl*MI polymorphism, primers CD5-F and either 1138wt-R or 1138mut-R were used to amplify a 619-bp fragment (fig. 2). PCR of the intron 10 *Pml*1 polymorphism used primers int10-R and either 1138wt-F or 1138mut-F to amplify a 473-bp fragment (fig. 2). Parental genomic DNA was amplified with either oligonucleotide primers CD5-F and 1138wt-R or int10-R and 1138wt-F (table 1). PCR reactions were performed with an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, 30 s at an annealing temperature of 60°C or 62°C, and an extension step for 30 s at 72°C. After amplification, the PCR products were digested with the appropriate restriction enzyme (*Pml*I or *Pfl*MI) and were analyzed by agarose-gel electrophoresis.

The polymorphic stretch of guanosine residues was analyzed by either of two methods: by direct sequencing of PCR products or by cloning of the PCR products and then sequencing (TA cloning; Invitrogen). Allele-specific PCR was used to amplify probands' genomic DNA corresponding to the normal or FGFR3 G1138A mutant allele, by use of both primer 1138wt-R or primer 1138mut-R and the CD5-F corresponding primer (table 1). Products from amplifications using the allele-specific primers were purified (Quiquick PCR Purification columns; Qiagen) and were sequenced. Parental genomic DNA was amplified by use of primers 1138wt-r and CD5-F (table 1). PCR products were cloned into the pCR-3 vector (Invitrogen), plasmid DNA was isolated, and the insert was sequenced directly to determine the phase of the polymorphic allele and the mutation.

Results

Determination of Polymorphisms

Three distinct polymorphisms were identified in the FGFR3 gene, within close proximity to the achondroplasia mutation site—two in intron 9 and one in intron

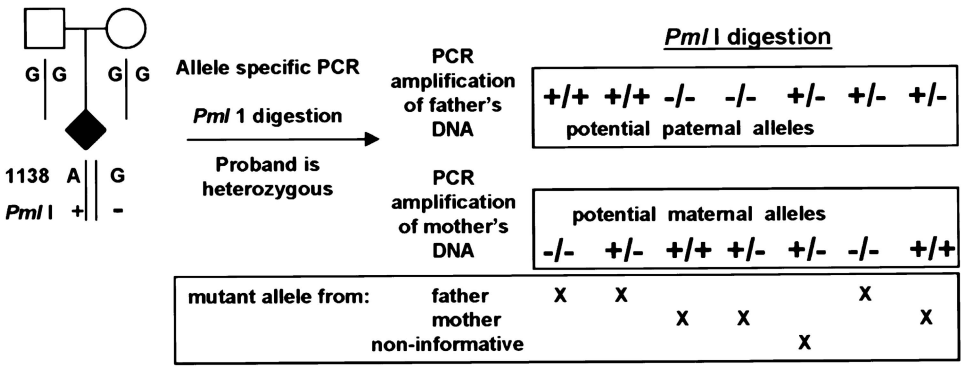

Figure 3 Potential paternal and maternal alleles. The *Pml*I polymorphism is used as an example. Allele-specific PCR was performed with the proband's genomic DNA, and digestion was performed with the appropriate restriction enzyme. If the proband was heterozygous for the polymorphism, the parental DNAs were analyzed. The possible parental alleles and the parent from whom the mutant allele would be inherited are shown. +/+ = Homozygosity for the polymorphic sequence, −/− = homozygosity for the normal sequence, and +/− = heterozygosity at the site.

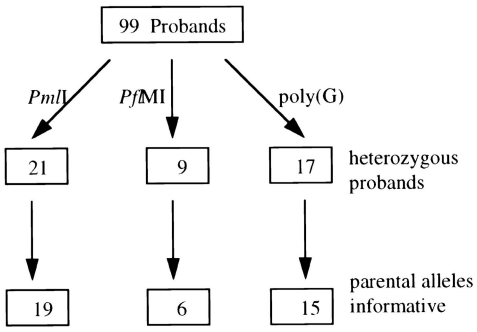

Figure 4 Analysis of 99 probands. A total of 21, 9, and 17 probands were heterozygous for the *Pml*I, *Pfl*MI, and poly(G) polymorphisms, respectively. From those heterozygous probands, respectively, 19, 6, and 15 parental alleles were informative for the determination of the parental origin of the achondroplasia allele. In all 40 informative families, the mutant allele was inherited from the father.

10. The polymorphisms included a G→C transition toward the 5′ end of intron 9, which created a *Pfl*MI site; a C→T transversion in intron 10, which created a *Pml*I site; and a single G deletion in a stretch of 11 consecutive guanosine residues in intron 9. To determine the frequency of the *Pfl*MI and *Pml*I polymorphisms in the general population, chromosomes of normal individuals were genotyped. Ten (4.5%) of 224 chromosomes carried the *Pfl*MI polymorphism, and 30 (10.4%) of 288 chromosomes carried the *Pml*I polymorphism.

For a family to be informative for any polymorphism, the proband must be heterozygous at the site (see results discussed below), with only one parent heterozygous for the polymorphism or both parents homozygous for opposing variants (fig. 3). Families in which the proband was homozygous for all three polymorphisms or in which both parents were heterozygous for the polymorphisms and in which the affected child also was heterozygous were noninformative. Ninety-one families were genotyped for the *Pml*I polymorphism, 83 were genotyped for the *Pfl*MI polymorphism, and 82 were genotyped for the poly(G) variant. Most families were typed for more than one polymorphism. Once an informative polymorphism had been found, no further analysis was done on that family.

Restriction Site–Polymorphism Analysis

ARMS was used to determine the inheritance pattern of specific alleles within the achondroplasia families. Allele-specific PCR was done with either the mutant chromosome or the normal chromosome. Digestion of the PCR product with either *Pfl*MI or *Pml*I distinguished the phase of the polymorphism and mutation (fig. 2). Twenty-one probands were heterozygous for the *Pml* I polymorphism, and nine probands were heterozygous for the *Pfl*M1 polymorphism.

A fragment of genomic DNA containing the RFLP site from the parents of these informative probands was amplified and digested with the appropriate restriction enzyme (fig. 2). Nineteen sets of parents were found to be informative for the *Pml* I restriction-site polymorphism, and six sets of parents were informative at the *Pfl*M1 site (figs. 3 and 4). In all 25 cases informative for the restriction-site polymorphisms, the proband's paternal chromosome carried the achondroplasia mutation.

Poly(G) Polymorphism Analysis

Sequencing of the PCR products determined both heterozygosity for the poly(G) polymorphism (fig. 5) and the phase of the polymorphism with respect to the achondroplasia mutation. Seventeen probands were determined to be heterozygous for the poly(G) polymorphism. Parents of these informative probands were genotyped at this site, and 15 sets of parents were found to

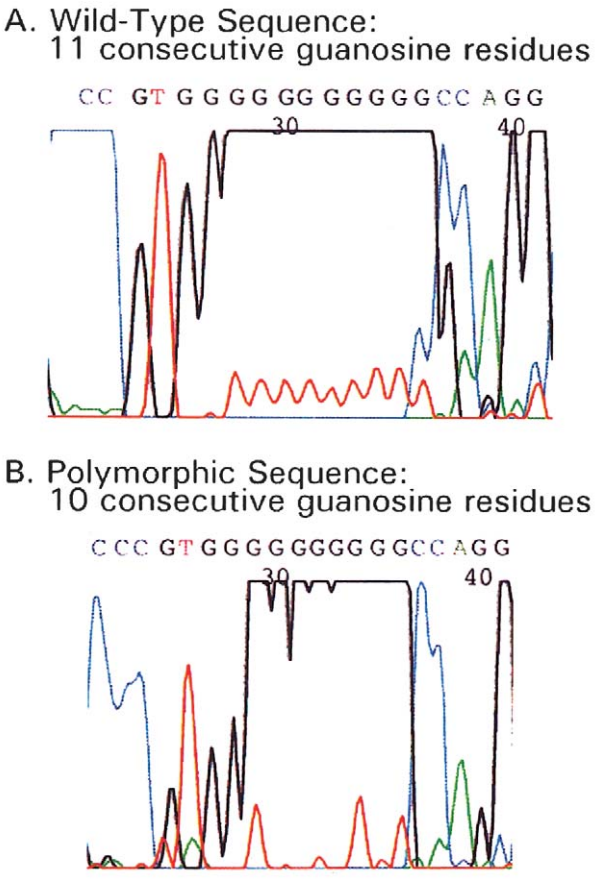

Figure 5 Sequence analysis of FGFR3 intron 9 poly(G) polymorphism. Normal intron 9 sequence (*A*) contains a stretch of 11 consecutive guanosine residues, whereas the polymorphic sequence (*B*) has a G deletion in this sequence.

be informative (fig. 4). In all 15 cases informative for the poly(G) polymorphism, the proband's paternal chromosome carried the achondroplasia mutation.

Discussion

Achondroplasia, the most common form of dwarfism, results predominantly from a specific mutation, G1138A, in the FGFR3 gene, with >97% of achondroplasia cases resulting from this mutation (Bellus et al. 1995*a*). The germ-line frequency of this mutation is estimated as being 5.5–28 $\times$ 10^{-6}, suggesting that it is the most mutable base in the human genome (Bellus et al. 1995*a*). Although this base change occurs in the context of a CpG dinucleotide, suggesting a spontaneous methylation-deamination mechanism for the generation of the mutation, the exceptionally high mutation rate of this nucleotide represents an intriguing biological phenomenon.

Using the ARMS method (Moloney et al. 1996), as well as allele-specific PCR followed by sequencing, we identified the parental origin of the mutant allele in 40 informative families. We have shown that, for new cases of achondroplasia, in the 40 informative families that we analyzed, the mutated allele was inherited exclusively from the father. Although this observation does not exclude inheritance of the achondroplasia allele from the mother, the incidence of the maternal allele carrying the achondroplasia mutation is probably low.

The mean paternal age for the achondroplasts analyzed in this study was 35.86 years, suggesting an advanced paternal–age effect. Achondroplasia was the first genetic disorder that was hypothesized to have a paternal-age component (Penrose 1955). More recently, paternal age also has been shown to be involved in other FGFR disorders, including thanatophoric dysplasia (Orioli et al. 1995) and Crouzon, Pfeiffer (Risch et al. 1987), and Apert syndromes (Moloney et al.1996). These are all disorders in which a few specific mutations result in occurrences of the disorder, raising the notion that the FGFR genes are predisposed to mutations at specific nucleotides, compared with what occurs in other genes.

Moloney et al. (1996) have demonstrated that, in 57 Apert syndrome families, the FGFR2 mutation arose from the paternal chromosome. Prior to their study, it had been observed that there was a tendency for the parents of Apert syndrome babies to be older than average (Blank 1960; Erickson and Cohen 1974), with the paternal age being more advanced than the maternal age. This led to Apert syndrome being associated with a specific paternal-age effect, with the syndrome having a less significant association with advanced maternal age. However, this association between a paternal-age effect and Apert syndrome was disputed, with the thought that maternal age contributed ~30% of the total effect (Risch et al. 1987). The results reported by Moloney et al. (1996) conclusively demonstrated that the parental-age effect is due to the father and that the postulated maternal contribution is small.

Male germ cells divide, after puberty, every 16 d. Thus, because of the significant number of meioses that they undergo, these cells may accumulate errors with advancing age. The advanced paternal–age effect that we have observed for new achondroplasia mutations suggests that these mutations arise during spermatogenesis in the unaffected father and that they neither are the result of somatic mosaicism in one of the parents nor occur postzygotically in the embryo, although we cannot rule out these mechanisms for the development of mutations that result in achondroplasia (Reiser et al. 1984). However, the lack of familial recurrence of achondroplasia would argue against significant levels of germ-line mosaicism, although there have been a few reports of multiple affected children from families with normal parents (Bowen 1974; Fryns et al. 1983; Reiser et al. 1984; Philip et al. 1988). Germ-line mosaicism in one parent is known to result in recurrence of other disorders, including osteogenesis imperfecta, which has a recurrence rate of ~7% (Cole and Dalgleish 1995), and pseudoachondroplasia (Ferguson et al. 1997).

FGFR3 nucleotide 1138, the site of the common achondroplasia mutation, occurs in the context of a CpG dinucleotide, predisposing this nucleotide to mutations. However, the abundance of the specific achondroplasia mutation, compared with other mutations at CpG dinucleotides, remains to be explained (Sommer 1995). The mutation rate for the G1138A achondroplasia mutation is enhanced by a factor of 10^2–10^3, compared with mutation rates at other CpG dinucleotides (Bellus et al. 1995*a*). It also is unclear why this particular mutation occurs so much more frequently during spermatogenesis than during oogenesis.

Finally, the high-frequency FGFR2 mutation that causes Apert syndrome does not occur in the context of a CpG dinucleotide (Moloney et al. 1996). Thus, despite the observations that both Apert syndrome and achondroplasia mutations occur exclusively on the paternally derived chromosome, the mechanism resulting in these FGFR mutations may not be the same. It is likely that other mechanisms, as yet unrecognized, are contributing to the high rates of mutation at specific sites in FGFR genes. We anticipate that studies aimed at dissecting the cause of high-frequency FGFR mutations, including the common achondroplasia mutation, will add substantially to our understanding of human mutational events.

Acknowledgments

The authors wish to thank Iain McIntosh, Ph.D., for helpful discussions; Margaret Abbott, R.N., for collecting clinical in-

formation; and Dr. Marek Sanak, for referring families. The authors also would like to thank the patients and their families for participating in this study. This study was supported, in part, by the Division of Intramural Research, National Human Genome Research Institute, National Institutes of Health; by European Union grant BMH1-CT93-1316; and by a grant from the Association Française des Myopathes.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for achondroplasia)

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