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Delayed bone age due to a dual effect of FGFR3 mutation in Achondroplasia

Stéphanie Pannier [a,b,1], Emilie Mugniery [a,1], Aurélie Jonquoy [a], Catherine Benoist-Lasselin [a], Thierry Odent [b], Jean-Philippe Jais [c], Arnold Munnich [a], Laurence Legeai-Mallet [a,*]

[a] INSERM U781-Université Paris Descartes-Hôpital Necker-Enfants Malades, 75015 Paris, France
[b] Service d'Orthopédie et Traumatologie Pédiatrique, Hôpital Necker-Enfants Malades, 75015 Paris, France
[c] Service de Biostatistique et Informatique Médicale, Hôpital Necker-Enfants Malades, 75015 Paris, France

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ABSTRACT

Achondroplasia (ACH), the most common form of human dwarfism is caused by a mutation in the Fibroblast Growth Factor Receptor 3 (FGFR3) gene, resulting in constitutive activation of the receptor. Typical radiological features include shortening of the tubular bones and macrocephaly, due to disruption of endochondral ossification. Consequently, FGFR3 has been described as a negative regulator of bone growth. Studying a large cohort of ACH patients, a delay in bone age was observed shortly after birth (for boys $p = 2.6 \times 10^{-9}$ and for girls $p = 1.2 \times 10^{-8}$). This delay was no longer apparent during adolescence. In order to gain further insight into bone formation, bone development was studied in a murine model of chondrodysplasia ($Fgfr3^{Y367C/+}$) from birth to 6 weeks of age. Delayed bone age was also observed in $Fgfr3^{Y367C/+}$ mice at 1 week of age followed by an accelerated secondary ossification center formation. A low level of chondrocyte proliferation was observed in the normal growth plate at birth, which increased with bone growth. In the pathological condition, a significantly high level of proliferative cells was present at birth, but exhibited a transient decrease only to rise again subsequently.
Histological and *in situ* analyses suggested the altered endochondral ossification process may result from delayed chondrocyte differentiation, disruption of vascularization and osteoblast invasion of the femur.
All these data provide evidence that FGFR3 regulates normal chondrocyte proliferation and differentiation during bone growth and suggest that constitutive activation of the receptor disrupts both processes. Therefore, the consequences of FGFR3 activation on the physiological process of bone development appear to be dependent on spatial and temporal occurrence. In conclusion, these observations support the notion that FGFR3 has a dual effect, as both a negative and a positive regulator of the endochondral ossification process during post-natal bone development.

Introduction

Achondroplasia (ACH) is the most common form of human dwarfism, and occurs with an estimated frequency of 1/15000. The diagnosis is usually made at birth [1]. Affected individuals exhibit short stature caused by rhizomelic shortening of the limbs, characteristic facies with frontal bossing and midface hypoplasia. The typical radiological features include shortening of the tubular bones, narrow interpediculate distance in the lumbar vertebra, short vertebral pedicules and decreased size of the base of the skull [1,2]. ACH is a fully penetrant autosomal dominant disorder, caused by a recurrent mutation (Gly380Arg) in the Fibroblast Growth Factor Receptor 3 (FGFR3) gene [3]. FGFR3 mutations also account for a milder phenotype, hypochondroplasia (HCH), [4] and the most severe form of dwarfism, thanatophoric dysplasia (TD) [5,6]. All FGFR3 mutations examined so far result in a ligand-independent phosphorylation of the tyrosine kinase domain [7]. The presentation of ACH signs has been ascribed to impaired epiphyseal growth. In vertebrates, most bones develop through a process known as endochondral ossification [8]. The growth plate consists of zones corresponding to the different stages of endochondral ossification, which evolve with age. Many growth factors, including bone morphogenic protein (BMPs), insulin-like growth factors and Wingless-int family members (WNTs) regulate the numbers of proliferating and hypertrophic chondrocytes [8]. Among them, there are two factors which can act together or independently: IHH (Indian Hedgehog) and PTHrP (parathyroid hormone-related protein) [9–13]. IHH, which is expressed in hypertrophic chondrocytes regulates PTHrP resulting in a negative feedback loop affecting differentiation. During fetal development and post-natal growth, cartilage is gradually replaced by bone via the development of a diaphyseal primary ossification center and an epiphyseal secondary ossification center. The formation of the secondary ossification center begins morphologically with the formation of the cartilage canal [14], which

* Corresponding author.
E-mail address: Laurence.legeai-mallet@inserm.fr (L. Legeai-Mallet).
[1] These authors contributed equally to this work.

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is associated with peripheral vascular proliferation that invaginates from the perichondrium. This formation involves cartilage degradation and mineralization, angiogenesis and finally bone matrix synthesis by osteoblasts [15]. This process begins at birth and continues throughout puberty resulting in an increased bone length.

Skeletal maturity or bone age is assessed radiographically and may be considered as corresponding to chronological age. Bone age is particularly useful in the clinical evaluation of children with growth and/or puberty disorders and is frequently used to predict the timing for limb lengthening and epiphysiodesis procedures. Skeletal dysplasias are constitutional diseases which are detected during the antenatal period, at birth or during infancy and differ in prognosis, inheritance patterns and pathogenetic mechanisms. Skeletal maturity in these diseases can be advanced or delayed and some fluctuations in the process of maturation are genetically controlled. Advanced bone age has been described in diseases such as e.g. diastrophic dysplasia (OMIM 222600) [16], Larsen syndrome (OMIM 150250) [17,18] or Blomstrand chondrodysplasia (OMIM 215045) [19]. While, some skeletal dysplasias are characterized by a delayed bone age, namely pseudoachondroplasia (OMIM 177170) [20,21] or cartilage hair hypoplasia (OMIM 250250) [22]. An Fgfr3 knock-out mouse model study suggests that FGFR3 negatively regulates bone formation [23]. To appreciate the role of FGFR3 during skeletal development, bone age of a large cohort of ACH patients was studied in a retrospective study using the Greulich and Pyle (GP) method [24]. A statistically significant bone age delay was found in both male and female ACH patients. In order to gain further insight into bone formation in humans, bone development was examined in a murine model of chondrodysplasia (*Fgfr3*$^{Y367C/+}$ mice) [25]. *Fgfr3*$^{Y367C/+}$ mice display a dwarfism characterized by short limbs, macrocephaly and an abnormal growth plate. These features resemble the phenotype observed in ACH. The assessment of bone age was studied in the inferior femoral epiphysis. After birth, a considerable delay in ossification was observed and was confirmed by histological analyses. Analysis of proliferating cell nuclear antigen (PCNA)-positive cells in the growth plate showed that proliferation was highly disrupted in the *Fgfr3*$^{Y367C/+}$ mice. These data were confirmed by cyclin D1 expression analysis. Cell proliferation was high at birth but decreased by 2 weeks. At later time points, an increased proliferation was again observed. IHH expression also varies during development in the *Fgfr3*$^{Y367C/+}$ mice, at birth expression is identical in control and *Fgfr3*$^{Y367C/+}$ mice while later, this expression is reduced in the mutant.

This study revealed several consequences of the FGFR3 mutation: disrupted proliferation, delayed chondrocyte differentiation in the growth plate with delayed vascularization and osteoblast invasion in the epiphyseal secondary ossification center. Although FGFR3 has been described as a negative regulator of bone growth, observations made in the control growth plate suggest that FGFR3 can function both as a negative and a positive regulator of bone during post-natal development. A dysfunction of chondrocyte proliferation and differentiation therefore results in bone age delay in *Fgfr3*$^{Y367C/+}$ mice and ACH patients.

Materials and methods

Achondroplasia subjects

The study group consisted of 148 children, recruited from the medical genetics department of the Necker-Enfants Malades hospital between 1960 and 2000. The ACH diagnosis was confirmed by clinical and radiographic examination. Most of the radiographs were obtained during the first consultation mostly in young ACH patients. The age and gender of each child were recorded and ranged from birth to 16 years for the 64 girls (mean age 4.4 years) and 84 boys (mean age 3.1 years). A collection of ACH radiographs was kindly provided by Dr Pierre Maroteaux (Necker-Enfants Malades hospital, Paris, France).

Bone age determination

The Greulich and Pyle (GP) method was used to determine bone age. This method is an atlas-matching method, in which an assessment of bone age is done by comparing the overall appearance of a left hand and wrist radiograph with the standard radiograph [24]. The radiographs from a total of 148 ACH children were blinded regarding the identity and age of the child prior to observer assessment. When multiple hand-wrist radiographs existed for individual children, the oldest radiograph was selected. Chronological age in years, months and days was calculated by subtracting the patient's birth date from the date on the radiography report. The method is however, partially subjective and may be susceptible to inter-observer variability. The radiographs were examined by three trained observers who independently determined the bone age for each radiograph.

Inter-observer agreement between two observers was assessed by Cohen's Kappa coefficient and Light's Kappa for three observers [26]. Confidence intervals were computed by Bootstrap re-sampling [27]. A Kappa coefficient <0.5 is generally considered to indicate a poor agreement, 0.5 to 0.75 a moderate one and >0.75, an excellent one [28]. Chronological age was compared with bone age based on radiographs, using the Wilcoxon signed rank test. Bone age delay was defined as the difference between bone and chronological ages. To study the dynamics of bone age, radiographs were compared to the chronological age over time. A non-parametric regression using smoothing splines was fitted between bone age delay and chronological age [29]. All the statistical computations were performed with the R V2.7 statistical software environment [30] and the corresponding packages (PSY, BOOT and GAM). A p-value <0.05 was considered as significant and confidence intervals (CI) were given at a 95% level.

Bone age determination in Fgfr3$^{Y367C/+}$ *mice*

The heterozygous mice (*Fgfr3*$^{Y367C/+}$) ubiquitously express the Y367C mutation and display a less severe dwarfism phenotype than human TD. Animals from a mixed background (C57BL/6j/129 Sv/Pas) survive for up to 8 weeks [25]. The mice were studied at birth (P0) and at five time points (P7, P14, P21, P28 and P42). The skeletons of the *Fgfr3*$^{Y367C/+}$ mice and their control littermates were radiographed on Kodak oncology Film using Cabinet X-ray System Faxitron series-Hewlett Packard (15 s, 35 KV and 3 mA). After X-ray analyses of the whole skeleton, bone age was determined postnatally solely based on the presence of ossification nuclei in the inferior epiphysis of the femur in *Fgfr3*$^{Y367C/+}$ mice and their control littermates. The analysis of femurs, rather than developing metacarpals allowed comparison of the radiographs with histological data. Knee joint radiographs of 35 *Fgfr3*$^{Y367C/+}$ mice and their control littermates (35 mice) were studied at several time points (P0-P42). Bone-stage development was assessed from the inferior epiphysis of the femur.

We defined a classification of standard radiographic images of the mouse joints. When present on the radiograph, an ossification nucleus was classified between 0 and 5: 0 = no ossification nucleus at the inferior epiphysis of the femur; 1 = initial appearance of an ossification nucleus; 2 = ossification nucleus $<15\%$; 3 = ossification nucleus size between 15% and 50%; 4 = ossification nucleus size $>50\%$ and 5 = 100% or complete ossification nucleus. A follow-up radiograph was performed during bone growth from birth to 6 weeks in 6 groups of mice pairs. All animal studies were conducted under an Animal study protocol approved by the animal Care and Use Committee.

Histological, in situ hybridization and immunological analyses

Femurs from 35 *Fgfr3*$^{Y367C/+}$ mice and their control littermates were isolated at P0, P7, P14, P21, P28, and P42. Some femurs were stained with Alizarin Red and Alcian Blue. Histological sections were prepared from femurs that were fixed in 4% paraformaldehyde,