Short-Limbed Dwarfism: slw Is a New Allele of Npr2 Causing Chondrodysplasia

CHIZURU SOGAWA, TAKEHITO TSUJI, YUSUKE SHINKAI, KENTARO KATAYAMA, AND TETSUO KUNIEDA

From the Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka, Okayama 700-8530, Japan (Sogawa, Tsuji, Shinkai, Katayama, and Kunieda). Chizuru Sogawa is now at the National Institute of Radiological Sciences, Inage, Chiba 263-8555, Japan; and Yusuke Shinkai is now at the Research Center for Health Science of Nanoparticles, Tokyo University of Science, Noda, Chiba 278-8510, Japan.

Address correspondence to T. Tsuji at the address above, or e-mail: takehito@cc.okayama-u.ac.jp.

Abstract

Short-limbed dwarfism (SLW) is a new mutant mouse characterized by a dwarf phenotype with markedly short body, limbs, and tail. In the present study, we investigated the skeletal phenotypes of the SLW mouse and determined the chromosomal localization to identify the gene responsible for the phenotypes (slw). Skeletal preparations stained with alcian blue and alizarin red revealed that longitudinal growth of the extremities of the affected (slw/slw) mice was significantly reduced in comparison with that of normal mice, whereas the positions and numbers of skeletal elements were normal. Histological examination of tibial growth plates of the affected mice showed that the numbers of proliferating and hypertrophic chondrocytes were obviously diminished. These phenotypes resembled to those of human chondrodysplasias caused by defective chondrocyte proliferation and differentiation. We mapped the slw locus on an 11.7-cM interval of the proximal region of mouse chromosome 4 by linkage analysis. Furthermore, allelism test using Npr2cn locus, a mutant allele of Npr2 gene encoding a natriuretic peptide receptor B, revealed that slw locus is an allele of the Npr2 gene. These results suggest that the dwarf phenotype of the SLW mouse is caused by the disturbed endochondral ossification, and a mutation in the Npr2 gene is expected to be responsible for the phenotypes of the SLW mouse.

The skeleton of vertebrates is formed by 2 different processes, intramembranous and endochondral ossifications. Intramembranous ossification forms the calvarium, mandible, and part of the clavicles, in which mesenchymal cells directly differentiate into osteoblasts. Endochondral ossification leads to the development of long bones that comprise the appendicular skeleton and vertebrae. During the endochondral ossification, mesenchymal cells initially differentiate into chondrocytes and progress through proliferating, maturing, and hypertrophic stages with strict columnar alignment. Distal hypertrophic chondrocytes undergo apoptosis and are replaced by trabecular bone. These processes of endochondral ossification are strictly controlled by many genes (Erlebacher et al. 1995), but the entire mechanism underlying the regulation of endochondral ossification remains obscure. Defective function of the genes regulating this process induces chondrodysplasias (Cohen 2002; Newman and Wallis 2003), which are a heterogeneous group of disorders characterized by abnormalities in cartilage and bone growth and development, often resulting in disproportionate dwarfism. Thus, understanding the genes responsible for chondrodysplasias should provide insight into the complex network of chondrogenesis in endochondral ossification.

Several spontaneous mouse mutations including ocd, cho, stb, bm, and cn exhibit disproportionate dwarfism (Lane and Dickie 1968; Seegmiller et al. 1971; Sweet and Bronson 1991). The causative mutations of cho, bm, and cn mice have been identified in genes encoding procollagen, type XI, alpha 1 (Li et al. 1995), 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Kurima et al. 1998), and natriuretic peptide receptor 2 (Tsuji and Kunieda 2005), respectively. Mutations in these genes have also been found in patients with human skeletal dysplasias (Williams and Jimenez 2003; Bartels et al. 2004). Therefore, mouse mutants showing chondrodysplastic phenotypes are useful animal models for investigating the etiologic factors involved in abnormal skeletal development in humans.

We have recently established a new mutant mouse named “short-limbed dwarfism” (SLW), which was characterized by a strikingly short body, limbs, and tail. In the present study, we analyzed the skeletal phenotypes of the SLW mouse and chromosomal localization of the locus for the SLW phenotype to identify the gene responsible for the SLW mouse.

Materials and Methods

Mice

An SLW founder male mouse was initially crossed with C57BL/6J female mice to produce F1 mice. Thereafter, F2
progeny were generated by intercrossing the F1 mice. Phenotypes of these F2 mice were determined at postnatal day 7 (P7) after birth. A total of 380 F2 progeny was obtained and used for linkage analysis. To establish an inbred strain, sister–brother mating between heterozygous mice was started from the F1 mice and has so far been continued for 11 generations. Because the inbreeding generations of the SLW mouse have not yet exceeded the 20 generations required for the establishment of an inbred strain, the mouse stock carrying the slw mutation is tentatively named SLW stock. The achondroplastic (Npr2cn/Npr2cn) mice obtained from The Jackson Laboratory were used for an allelism test.

Body Length

Body length was measured as the length from the nose to the anus of female affected and normal mice every 3 days until P21 and then weekly until P84. Mice born in litters of 5–7 pups were used for this measurement. All mice were weaned at P21 and then reared in groups of 4–6 per cage with free access to food and water. All data are expressed as the means ± SD, and the statistical significance of differences was determined using Student’s t-test.

Skeletal Preparation

The skin and internal organs of affected and normal littermate mice were removed. The skeletons were fixed in 95% ethanol for 1 day and then stained with 0.15% alcin blue in 80% ethanol and 20% acetic acid for 1 day. Fixed skeletons were dehydrated in 100% ethanol and immersed in 2% KOH for 1–7 days. The skeletons were then stained with 0.015% alizarin red in 1% KOH for 1 day, cleaned in a series of graded glycerin, and stocked in glycerin and ethanol (1:1).

Histological Examination

The affected and normal littermates at P7 and P21 were killed under anesthesia and then the tibias were fixed in 4% paraformaldehyde overnight at 4 °C. After decalcification in 10% ethylenediaminetetraacetic acid for 2–12 days, the specimens were dehydrated and embedded in paraffin. Tibial growth plates were sectioned at 5 μm and stained with hematoxylin and eosin.

Linkage Analysis

Sixty-nine mutant progeny of the 380 F2 mice obtained from cross between SLW and C57BL/6J mice were used for linkage analysis. Genomic DNAs were prepared from the liver of F2 mice by phenol–chloroform extraction. The genotype of the microsatellite markers covering all mouse chromosomes was determined by polymerase chain reaction (PCR) with 35 cycles at 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 45 s. PCR was carried out in 10 μl of reaction mixture containing 1× PCR buffer, 0.2 mM dNTP, 0.5 μM of each primers, and 0.25 U Tag DNA polymerase (Amersham Biosciences, Piscataway, NJ). PCR products were electrophoresed on 3.0% agarose gels and stained with ethidium bromide. These genotyping data were analyzed for linkage between the dwarf phenotype and these microsatellite markers using Map Manager QTL software.

Allelism Test

An allelism test of slw with the Npr2 gene was performed by reciprocal cross between slw heterozygote mice and Npr2 mice.

Results

Mating Experiments

The SLW mouse originated from a male mouse showing a dwarf phenotype with short limbs and a tail found in the ddY mouse colony maintained at Okayama University. This male mouse was mated with female C57BL/6J mice and obtained 8 F1 offspring (4 females and 4 males), which showed no phenotypic abnormality. Intercross between these F1 mice generated affected and normal F2 progeny. The numbers of the F2 progeny at P7 were 380 consisting of 64 affected (41 females and 23 males) and 316 normal (162 females and 154 males) mice. The ratio of affected to normal mice at P7 was significantly (P = 0.001) lower than that expected from Mendelian inheritance of an autosomal single recessive trait (Table 1). In particular, distortion of the ratio between affected and normal mice was more obvious in males than females. Mating between affected males and normal females generated 8 affected and 6 normal mice, and mating between both affected males and females yielded 3 affected and no normal mice. Furthermore, none of the 316 normal F2 mice showed homozygosity of the haplotype of the microsatellite markers associated with the expected mutant allele, indicating that the phenotype of SLW mouse presumably does not appear in heterozygous (slw+/+) mouse. These results suggested that the phenotype of the SLW mouse is controlled by an autosomal single recessive gene with full penetrance, but a significant number of the affected mice died during gestation or after birth. We named the locus responsible for the SLW phenotypes slw.

Gross Morphological Observation

Although affected SLW mice are not apparently different from normal mice at the newborn stage, they are
distinguishable by a slightly dome-shaped skull with a short stature and tail at P7. These abnormalities become progressively more obvious as the mice grow (Figure 1A). Measurement of the body length indicated that the length of the affected mice did not significantly differ from that of normal littermates at P0 but became significantly shorter by P3 ($P < 0.02$). The differences of the body lengths were more obvious during P6–84 ($P < 0.0002$), and the average length of the affected mice was 70–80% of that of normal littermates (Figure 1B).

To examine the gross skeletal feature of SLW mice, we stained the skeletons of affected and normal mice at P7, P21, and P84 with alcian blue and alizarin red. At 1 and 3 weeks, the staining patterns of cartilage and calcified bones of affected and normal mice showed no obvious difference (data not shown). The positions and numbers of skeletal elements of affected mice were normal. However, all skeletal bones formed by endochondral ossification were smaller in affected mice than in normal littermates. In particular, the appendicular bone was obviously shorter than that of normal mice (Figure 2A). The femur and humerus were more severely affected than the tibia, fibula, radius, and ulna (Figure 2A and B). In addition to these deformities of the appendicular and axial skeletons, the craniofacial bones of the affected mice showed morphological abnormalities including dome-shaped skulls (Figure 2C). The skulls of the affected mice were reduced in size along the anterior–posterior axis, but dorsal–ventral and left–right axes were slightly longer than normal mice. The supraoccipital bones of the affected mice were smaller and the nasal length was also slightly reduced. Conversely, the parietal and interparietal bones were slightly larger than those of normal mice (Figure 2C). No other gross abnormalities in skeletal morphological were observed in affected mice.

**Histological Analysis**

To further analyze defective longitudinal bone growth in SLW mice, we performed histological analyses by staining tibial growth plates with hematoxylin–eosin at P7 and P21. At P7, the epiphyseal growth plate was slightly narrower in affected mice than in normal mice (Figure 3A and B). The numbers of proliferating and hypertrophic chondrocytes were decreased in affected mice, and the hypertrophic zone was obviously narrow. Furthermore, the height of hypertrophic chondrocytes was slightly reduced (Figure 3C and D). At P21, the growth plates of the affected mice were
obviously narrower than those of normal littermates. The proliferating and hypertrophic zones of the affected mice were obviously reduced (Figure 3E and F), whereas resting chondrocytes at P7 and P21 were normal. These histological findings indicated that the process of the proliferation and differentiation of chondrocytes during endochondral ossification are impaired in affected SLW mice. These pathological features of the SLW mouse most resemble those of human chondrodysplasias showing disturbed endochondral ossifications. We, therefore, concluded that the skeletal phenotype of the SLW mouse is characterized as chondrodysplasia.

**Linkage Analysis**

We investigated the chromosomal localization of the slw locus by linkage analysis with homozygous (slw/slw) F<sub>2</sub> progeny generated by intercrossing heterozygous (slw/+) F<sub>1</sub> mice. The genotypes of microsatellite markers positioned at approximately 20-cM intervals on entire mouse chromosomes were determined in F<sub>2</sub> progeny. Linkage between the slw locus and these microsatellite markers was analyzed by Map Manager QTL software, and significant linkage was observed with markers on the proximal region of mouse chromosome 4. We further examined the precise linkage using additional markers located on this region. No recombination was found between the slw locus and D4Mit109 marker. The order and distances between the slw locus and microsatellite markers were as follows: D4Mit172—(5.1 ± 1.9 cM)—D4Mit109, slw—(6.6 ± 2.1 cM)—D4Mit139 (Figure 4A and B); therefore, the slw locus was mapped in an approximately 11.7-cM interval of the proximal region of mouse chromosome 4.

**Allelism Test**

In the 11.7-cM interval of the slw locus, several candidate genes including the Npr2 gene encoding a selective receptor for C-type natriuretic peptide (CNP) have been localized (National Center for Biotechnology Information mouse genome database http://www.ncbi.nlm.nih.gov/). Because mutations of this gene in humans and mice have been demonstrated to cause a dwarf phenotype (Bartels et al. 2004; Tamura et al. 2004; Tsuji and Kunieda 2005), the Npr2 gene was the most possible candidate gene for the SLW mouse. To evaluate whether or not the Npr2 gene is involved in the dwarf phenotype of the SLW mouse, we performed an allelism test of the slw with Npr2<sup>cn</sup> allele, which possesses a loss-of-function mutation in the Npr2 gene (Tsuji and Kunieda 2005). Mating between slw heterozygote mice and Npr2<sup>cn</sup> heterozygote mice gave 16 offspring, and 6 of these mice showed dwarf phenotype similar to gross skeletal feature of the Npr2<sup>cn</sup>/Npr2<sup>cn</sup> and slw/slw mice (Table 2). Because heterozygous mice of neither slw nor Npr2<sup>cn</sup> exhibit the skeletal abnormality, the results of the allelism test suggested that the slw locus is a mutant allele of the Npr2 gene.

**Discussion**

In the present study, the skeletal phenotype of the SLW mouse was characterized as chondrodysplasia showing disturbed endochondral ossification caused by defective chondrocyte proliferation and differentiation. Chondrodysplasia is the most common skeletal anomaly in humans, characterized by growth retardation and disproportionate dwarfism caused by disturbed endochondral ossification. In the endochondral ossification, the sequential and synchronous processes of chondrocyte proliferation and differentiation are essential and are tightly regulated by a number of local and systemic factors (Erlebacher et al. 1995). Several
Allelism test between slw and Npr2

<table>
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<tr>
<th>slw/+ × Npr2&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Normal</th>
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<td>4 (♀2 and ♂2)</td>
<td>3 (♀2 and ♂1)</td>
<td>7</td>
</tr>
<tr>
<td>Npr2&lt;sup&gt;+/−&lt;/sup&gt; × slw/+</td>
<td>6 (♀4 and ♂2)</td>
<td>3 (♀2 and ♂1)</td>
<td>9</td>
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We mapped the slw locus on the 11.7-cM region of mouse chromosome 4 by linkage analysis. Among the genes located in this region, Rmp1, Tgfr1, and Npr2 were predicted to be candidate genes for the SLW mouse. The Rmp1 encodes the RNA component of the mitochondrial RNA-processing endonuclease (Chang and Clayton 1989). A mutation in the human homologous gene, RMRP, causes cartilage–hair hypoplasia, an autosomal recessive chondrodysplasia with severe short-limbed short stature (Ridanpaa et al. 2001). Tgfr1, the gene for type I receptor of transforming growth factor-beta, is expressed in resting, maturating, and hypertrophic chondrocytes and regulates the rate of differentiation to hypertrophic chondrocytes (Serra et al. 1999). The Npr2 gene encodes a natriuretic peptide receptor B (NPRB), which is a receptor for CNP. The CNP is known to stimulate longitudinal bone growth by increasing the proliferation and hypertrophy of chondrocytes and by producing cartilage matrix (Yasoda et al. 1998; Mericq et al. 2000). Mice deficient for CNP (Npp<sup>e</sup>/e) exhibit dwarfism due to a reduced zone of proliferating and hypertrophic chondrocytes (Chusho et al. 2001). Furthermore, we (Tsuiji and Kunieda 2005) and another group (Tamura et al. 2004) have recently revealed that deficiencies of Npr2 gene in mice also cause the dwarf phenotype with disturbed endochondral ossification, which morphologically and histologically resemble those of the SLW mouse. Thus, the Npr2 gene was predicted to be the most potent candidate gene for the SLW mouse.

The allelism test using a mating between slw/+ mice and Npr2<sup>+/−</sup> mice showed that slw was an allele of the Npr2 gene. Npr2<sup>−/−</sup> locus contains a missense mutation resulted in lack of the guanyl cyclase activity of NPRB, by which the ability to produce an intracellular second messenger, cyclic guanosine monophosphate, of NPRB has been disrupted (Tsuiji and Kunieda 2005). Therefore, slw would also contain a loss-of-function mutation in the Npr2 gene. Identification of a mutation in the Npr2 gene of the SLW mouse is necessary to confirm this possibility.

It is to be noted that there were some differences between the phenotypes of the Npr2 gene–deficient mice and the SLW mice. For example, the Npr2 gene–deficient mice showed abnormalities in reproductive systems including ovarian function (Tamura et al. 2004; Kiyosu C, Tsui T, Kunieda T, unpublished data), whereas the SLW mice have no reproductive abnormalities. These might be due to the differences of genetic background of mouse strain or different types of mutations, and the SLW mouse would be a unique tool to analyze the roles of Npr2 gene in reproductive system as well as in skeletal formation.

In particular, because acromesomelic dysplasia Maroteaux type (AMDM) is an autosomal recessive human disorder characterized by disproportionate short stature and shortening of the extremities caused by mutations in the Npr2 gene (Bartels et al. 2004), the SLW mouse would be a useful model for investigating the pathogenesis and therapeutic approaches of AMDM.
References


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