An Overgrowth Disorder Associated with Excessive Production of cGMP Due to a Gain-of-Function Mutation of the Natriuretic Peptide Receptor 2 Gene

Kohji Miura¹, Noriyuki Namba¹, Makoto Fujiwara¹, Yasuhisa Ohata¹, Hidekazu Ishida¹, Taichi Kitaoka¹, Takuo Kubota¹, Haruhiko Hirai¹, Chikahisa Higuchi², Noriyuki Tsumaki³, Hideki Yoshikawa², Norio Sakai¹, Toshimi Michigami⁴, Keiichi Ozono¹*

¹ Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, ² Department of Orthopedics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, ³ Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Kyoto, Japan, ⁴ Department of Bone and Mineral Research, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan

Abstract

We describe a three-generation family with tall stature, scoliosis and macrodactyly of the great toes and a heterozygous p.Val883Met mutation in Npr2, the gene that encodes the CNP receptor NPR2 (natriuretic peptide receptor 2). When expressed in HEK293A cells, the mutant Npr2 cDNA generated intracellular cGMP (cyclic guanosine monophosphate) in the absence of CNP ligand. In the presence of CNP, cGMP production was greater in cells that had been transfected with the mutant Npr2 cDNA compared to wild-type cDNA. Transgenic mice in which the mutant Npr2 was expressed in chondrocytes driven by the promoter and intronic enhancer of the Col11a2 gene exhibited an enhanced production of cGMP in cartilage, leading to a similar phenotype to that observed in the patients. In addition, blood cGMP concentrations were elevated in the patients. These results indicate that p.Val883Met is a constitutive active gain-of-function mutation and elevated levels of cGMP in growth plates lead to the elongation of long bones. Our findings reveal a critical role for NPR2 in skeletal growth in both humans and mice, and may provide a potential target for prevention and treatment of diseases caused by impaired production of cGMP.

Introduction

Several lines of evidence indicate that signaling triggered by CNP plays an important role in chondrocyte development [1,2]. Upon CNP binding, its cognate receptor natriuretic peptide receptor 2 (NPR2) functions as a guanylyl cyclase to increase cyclic guanosine monophosphate (cGMP) levels in chondrocytes, female reproductive organs, and endothelial cells [3,4]. Transgenic mice that overproduce CNP exhibit excessive growth, while defects of the CNP or Npr2 gene, leading to impairment of skeletal development [5–7]. The increase in cGMP level activates cGMP-dependent protein kinase II and seems to promote the accumulation of extracellular matrix in the growth plate of CNP-transgenic mice [8]. In human, overproduction of C-type natriuretic peptide (CNP) due to a chromosomal translocation was reported to cause skeletal dysplasia associated with tall stature [9–10]. In addition, acromesomelic dysplasia, type Maroteaux, characterized by dwarfism and short limbs, is caused by loss-of-function mutations in the Npr2 gene [11]. On the other hand, NPR3, which is thought to act as a clearance receptor, knock-out mice resemble CNP transgenic mice [12].

In this paper, we describe the first family with tall stature and macrodactyly of both great toes caused by a gain-of-function type mutation in the Npr2 gene. The mutant receptor, p.Val883Met, constitutively generates cGMP in vitro. Animal studies using the transgenic mice expressing the mutant NPR2 in chondrocytes demonstrated that skeletal overgrowth was associated with the overproduction of cGMP in cartilage. Our findings provide evidence that cGMP production downstream CNP/NPR2 system regulates the proliferation and differentiation of chondrocytes and determines skeletal growth.

Results

Case Description

The proband was a 15-year-old boy born to non-consanguineous parents. He was born at 40 weeks gestation by spontaneous labor after an uneventful pregnancy. Birth parameters were as follows: weight, +0.78 SD; length, +3.1 SD; and head circumference; −0.2 SD. Long and wide great toes were already present at birth. His height remained above +2 SD (Fig. 1A). His psychomotor development was normal for his age. Fractures at the age of 11 and 12 years led to his referral to our unit. At that time, his height was 177.0 cm (+2.7 SD). His weight and arm span were in the normal range, +0.8 SD and 99.7% of height, respectively, and his stature was proportional. Blood pressure was normal. Physical examination...
showed tall stature, scoliosis, and long hands and feet with arachnodactyly of all the fingers and toes, the great toes being markedly long and wide. The fifth distal phalanx of the hand also showed minor clinodactyly. He has given written informed consent to publication of his photographs. The right foot of the mother and grandmother, respectively. The mother’s great toes were surgically shortened at 15 years of age. The grandmother’s great toes were long and wide (G), and the fifth distal phalanx of the hand showed minor clinodactyly (H). Radiographs of the mother. Severe scoliosis and lumbar vertebra fractures (I) as well as a markedly long and wide great toe (J), and minor clinodactyly in the fifth digit of the hand (K) were observed, like in the proband. (L) Family tree. III-1, II-5, and I-3 indicate the proband, mother, and maternal grandmother, respectively, who had the phenotype. I-2 indicates a maternal grand-aunt, who had no phenotype and was included in the present study.

doi:10.1371/journal.pone.0042180.g001

Figure 1. Clinical manifestations of the family. (A) Growth curve of the proband. The X- and Y- axis indicate age (year) and height (cm) or weight (kg), respectively. The bold line represents the mean for Japanese males. (B, C) Photographs of the proband’s right foot (B) and hand (C). The great toe was markedly long and wide. The fifth distal phalanx of the hand also showed minor clinodactyly. He has given written informed consent to publication of their photographs. (D, E) Photographs of the mother (D) and grandmother (E), respectively. The mother’s great toes were surgically shortened at 15 years of age. The grandmother’s great toes were long and wide (G), and the fifth distal phalanx of the hand showed minor clinodactyly (H). Radiographs of the proband’s skeleton. His spine showed mild scoliosis (F). The great toe was markedly long and wide (G), and the fifth distal phalanx of the hand showed minor clinodactyly (H). Radiographs of the mother. Severe scoliosis and lumbar vertebra fractures (I) as well as a markedly long and wide great toe (J), and minor clinodactyly in the fifth digit of the hand (K) were observed, like in the proband. (L) Family tree. III-1, II-5, and I-3 indicate the proband, mother, and maternal grandmother, respectively, who had the phenotype. I-2 indicates a maternal grand-aunt, who had no phenotype and was included in the present study.

doi:10.1371/journal.pone.0042180.g001

The family tree (Fig. 1L) illustrates that the proband [III-1] has no siblings, and that his mother [II-5] and maternal grandmother [I-3] have the same phenotype (Fig. 1D, E). Clinical manifestations of II-5 were as follows: age, 46 years; height, 176 cm (+3.8 SD for her age); blood pressure, 140/70 mmHg. She had a history of hypertension and was not given medication, including antihypertensive drugs. Her menstrual cycles...
are regular. Radiological examination of II-5 showed severe scoliosis and lumbar vertebra fractures as well as markedly long and wide great toes, and minor clinodactyly in the fifth digit of the hands, like the proband (Fig. 1I–K). The BMD of the lumbar spine for L2–4 could not be evaluated due to compressed fractures of the lumbar vertebrae. Clinical manifestations of I-3 were as follows: age, 63 years; height, 166 cm (+3.0 SD for her age); blood pressure, 134/70 mmHg. She underwent right hip replacement arthroplasty at 50 years of age because of degenerative hip disease. She developed hypertension up to 210/110 mmHg at 59 years of age, when antihypertensive therapy was initiated. Her great toes were large in early life and shortened due to arthrogryposis with aging.

Identification of a Novel Missense Mutation p.Val883Met in the Npr2 Gene

Since the phenotype of the patients most closely resembled several cases of the CNP overproduction phenotype due to a chromosomal translocation, in terms of tall stature and large great toes [9–10], enhanced CNP/NPR2 signaling was suspected. The concentration in serum of amino-terminal pro CNP was slightly decreased in III-1; 3.02 ± 1.15 pmol/l, compared to the control (7.54 ± 1.50 pmol/l). Those in II-5 and I-3 were almost in the normal range; 2.29 and 4.99 pmol/l, respectively, compared to the control subjects (3.53 ± 0.75 pmol/l), ruling out overproduction of CNP. Since the proband’s phenotype also showed similarity to CATSHL syndrome [13], caused by a loss-of-function mutation in the Fgfr3 gene, except for the absence of neurological symptoms, the Fgfr3 gene was analyzed as well as the natriuretic peptide precursor C (Nppc), Npr2, and Npr3 genes.
We screened these four genes in III-1, II-5, I-3, and a maternal grand-aunt [I-2] who had no phenotype using genomic DNA extracted from peripheral blood leukocytes, and identified a novel heterozygous G→A missense mutation at nucleotide +2647 (c.2647G→A) of the Npr2 gene in III-1, II-5, and I-3 that creates a substitution of methionine for valine (p.Val883Met), while the same position in I-2 was not mutated (Fig. 2A). This variant was not registered in the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/) databases nor found in 214 alleles from healthy Japanese controls. Amino acid Val883 is located in a highly conserved region of the carboxyl-terminal guanylyl cyclase domain of NPR2 and is conserved across species (Fig. 2B). No mutations were found in the Fgfr3, Nppc, and Npr3 genes.

Increased Plasma cGMP Concentrations in the Patients

The plasma concentrations of cGMP in 10 healthy adults were 3.1±1.1 pmol/ml. Plasma cGMP concentrations were elevated in III-1, II-5, and I-3: 20.0, 12.0, and 7.3 pmol/ml. Conversely, that in I-2 without the phenotype was 3.6 pmol/ml, which was similar to the values in healthy adults.

p.Val883Met is a Gain-of-Function Mutation

To investigate the pathogenic significance of the p.Val883Met mutation, an in vitro functional assay was performed. HEK293A cells were transfected with HA-WT or HA-Val883Met constructs, and lysates were harvested for Western blotting using anti-HA antibody. As an internal control, β-actin in each sample was detected with anti-β-actin antibody. (B) Increased cGMP production in the HEK293A cells transfected with the p.Val883Met mutant (Mut) compared to that in wild-type cells (WT). Forty-eight hours after the transfection, the cells were serum-starved for 24 h, and then treated with the indicated concentrations of CNP-22 for 10 min, before cGMP production was assayed. Results are presented as the mean ± SD (N = 3, *p<0.05).

doi:10.1371/journal.pone.0042180.g003

Figure 3. p.Val883Met is a gain-of-function mutation. (A) Western blot analysis confirmed the comparable expression of HA-WT (WT) and HA-Val883Met (Mut). HEK293A cells were transfected with HA-WT or HA-Val883Met constructs, and lysates were harvested for Western blotting using anti-HA antibody. As an internal control, β-actin in each sample was detected with anti-β-actin antibody. (B) Increased cGMP production in the HEK293A cells transfected with the p.Val883Met mutant (Mut) compared to that in wild-type cells (WT). Forty-eight hours after the transfection, the cells were serum-starved for 24 h, and then treated with the indicated concentrations of CNP-22 for 10 min, before cGMP production was assayed. Results are presented as the mean ± SD (N = 3, *p<0.05).

We screened these four genes in III-1, II-5, I-3, and a maternal grand-aunt [I-2] who had no phenotype using genomic DNA extracted from peripheral blood leukocytes, and identified a novel heterozygous G→A missense mutation at nucleotide +2647 (c.2647G→A) of the Npr2 gene in III-1, II-5, and I-3 that creates a substitution of methionine for valine (p.Val883Met), while the same position in I-2 was not mutated (Fig. 2A). This variant was not registered in the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/) databases nor found in 214 alleles from healthy Japanese controls. Amino acid Val883 is located in a highly conserved region of the carboxyl-terminal guanylyl cyclase domain of NPR2 and is conserved across species (Fig. 2B). No mutations were found in the Fgfr3, Nppc, and Npr3 genes.

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p.Val883Met is a Gain-of-Function Mutation

To investigate the pathogenic significance of the p.Val883Met mutation, an in vitro functional assay was performed. HEK293A cells were transfected with HA-WT or HA-Val883Met constructs, and lysates were harvested for Western blotting. The Western blot analysis using anti-HA antibody confirmed that HA-WT and HA-Val883Met were expressed at comparable levels, with an approximate molecular size of 120 kDa (Fig. 3A). cGMP production in the cells expressing HA-WT and HA-Val883Met were also measured (Tg. 28, 102.2×10^-6 M increased intracellular cGMP levels 6.3 times higher in HA-Val883Met-expressing cells than HA-WT-expressing cells (*p<0.05) (Fig. 3B). Intriguingly, cGMP was produced in HA-Val883Met-expressing cells even in the absence of CNP, while no production was observed in HA-WT-expressing cells. These results indicate that p.Val883Met is a gain-of-function type mutation.

Transgenic Expression of the p.Val883Met Mutant Npr2 in Mice Resulted in a Skeletal Phenotype Similar to that Observed in the Patients

Then, we further investigated the effects of p.Val883Met on skeletogenesis by generating transgenic mice expressing the mutant Npr2 in chondrocytes. Transgenic founder No. 4 exhibited severe bone deformities including elongation of the spine with severe kyphosis, metatarsal bones, and distal phalanges, and died at seven weeks (Fig. 4A). Founder No. 28 also exhibited an elongated tail and toes with mild kyphosis, and could produce offspring mice. The amount of transgene mRNA in cartilage was quantified by real-time PCR and standardized based on the mRNA level of Sox9, a marker for chondrocytes. The higher expression of the transgene mRNA was detected in No. 4 than in No. 28 (data not shown).

Then, we evaluated the skeletal phenotype in No. 28 offspring (Tg. 28). First, the expression of Npr2 was determined using RNA extracted from costal cartilage of Tg. 28 and wild-type littermates (Wt) at 5 days of age. The total amount of Npr2 mRNA, including native and transgenic Npr2, was quantified by real-time PCR and standardized based on the mRNA level of Gapdh (Tg. 28, 1.83±0.77; Wt, 0.36±0.08, *p<0.05) (Fig. 4B). cGMP concentrations in costal cartilage were also measured (Tg. 28, 102.2±11.3; Wt, 5.9±0.6 pmol/mg DNA, *p<0.05) (Fig. 4C). Although the total amount of Npr2 was 5.1 times higher in Tg. 28 than Wt, the cGMP concentration was 17.3 times higher, supporting the in vitro data demonstrating the gain of function of the p.Val883Met mutant NPR2.
Representative radiographs of Tg. 28 (female, 8-week-old) are shown in Fig. 4D–G. The spinal and tail vertebrae and phalanges were longer in the transgenic mouse than Wt, indicating the longitudinal overgrowth of bones. Body length (data not shown) and tail length (Fig. 4H) were significantly longer in Tg. 28 than Wt from 8 to 18 weeks. In adulthood, Tg. 28 came to exhibit severe kyphosis and claudication in the legs, resembling the symptoms observed in patient I-3 and transgenic founder No. 4. In addition, naso-occipital length of the calvarium, femoral length, and tibial length were significantly longer in Tg. 28 than Wt at 18 weeks (*p<0.05) (Fig. 4I). Thus, the transgenic mice exhibited the skeletal overgrowth associated with the overproduction of cGMP in cartilage.

**Transgenic Mice Expressing the p.Val883Met Mutant Npr2 Exhibited Wider Growth Plates than Wild-type Littermates**

Histological examination of the femora and tibiae in 8-week-old mice revealed that Tg. 28 had wider growth plates than Wt.
(Fig. 5A). At higher magnification, a disorganized arrangement of chondrocytes with increased number of hypertrophic chondrocytes was observed in Tg. 28 (Fig. 5B).

**Discussion**

Here we have described a three-generation family with tall stature and macrodactyly due to a newly identified gain-of-function mutation of the \( \text{Npr2} \) gene, p.Val883Met. Increased levels of cGMP both in blood samples of the patients and whole cell lysates expressing the mutant receptor clearly indicate its hyperphosphorylation. Transgenic mice in which p.Val883Met mutant NPR2 was expressed in chondrocytes exhibited the elevated cGMP concentration and the excessive growth and deformities of vertebrae and long bones, which reproduced the symptoms observed in the patients. Taken together, our data are compatible with the interpretation that p.Val883Met mutant is constitutively active and causes skeletal overgrowth by increasing the level of cGMP in chondrocytes. However, we cannot exclude the possibility that the high expression level of NPR2 also contributed to overgrowth in transgenic mice to some extent.

Histological examination confirmed that the skeletal overgrowth was caused by the widening of the growth plates in the transgenic mice expressing the mutant \( \text{Npr2} \) (Fig. 5). As expected, the histological finding was similar to, but severer than, that reported in the mice with overexpression of CNP in chondrocytes [14]. The disorganized arrangement of chondrocytes in the transgenic mice indicates the critical role of the CNP/NPR2 pathway in chondrogenesis. Interestingly, the number of hypertrophic chondrocytes appeared to be increased in the transgenic mice,

![Figure 5. Hematoxylin-eosin-stained sections of growth plates of Tg. 28 and Wt at 8 weeks of age.](image)

The magnifications are \( \times40 \) (scale bar = 100 nm) and \( \times200 \) (scale bar = 50 nm) in (A) and in (B), respectively. doi:10.1371/journal.pone.0042180.g005

**Table 1.** Primer sets for the human \( \text{Nppc} \) gene.

| Exon | Forward sequence (5’→3’) | Reverse sequence (5’→3’) | Product size (bp) |
|------|--------------------------|--------------------------|------------------|
| 1    | GATTATAAGGCAGCGAGCAG     | CTGCTTCCCCCTCTCCTCCTG    | 366              |
| 2    | CTGGGAGGGGACACCC         | CACAACTTGAGCAAGCAGG      | 517              |

doi:10.1371/journal.pone.0042180.t001
suggesting the involvement of CNP/NPR2 pathway in chondrocyte differentiation.

Although NPR2 is expressed in various tissues, the phenotype seems to be confined to cartilage and bone in humans, as suggested by the clinical manifestations of our patients. It is worth noting that the transmission of the mutant Npr2 gene seems to have had no apparent effect on fertility in the family, although infertility was observed in Nppc or Npr2 null or hypomorphic mice [7,15].

The second messenger cGMP is generated by two distinct types of guanylyl cyclases; cytoplasmic (soluble) and membrane-bound [16]. The two types share a similar catalytic domain which is conserved from bacteria to humans [17–18]. Seven mammalian membrane-bound guanylyl cyclases have been identified, consisting of ligand-binding, kinase homology, dimerization, and guanylyl cylase domains [4]. ANP, BNP, CNP, and intestinal peptides such as guanylin and uroguanylin have been identified as ligands for the membrane-bound guanylyl cylases. Although a few constitutively activate guanylyl cyclases were synthesized by chemical methods [19], p.Val883Met is the first gain-of-function mutation of NPR2 in living beings. Since cGMP is an important second messenger of several bioactive factors, cGMP-related drugs such as inhibitors of cyclic nucleotide phosphodiesterase have been developed. Erectile dysfunction, pulmonary dysfunction, cardiomyopathy, headache, dementia, and cancer are the targets of treatment with drugs that increase cGMP levels. However, drugs that directly activate guanylyl cylases have not been developed, with the exception of BAY 63-2521 (riociguat) [20]. The amino acid Val883 is located in the catalytic domain and preserved across species. The mechanism of activation in the mutant is beyond the scope of this paper, but the substitution may facilitate a conformational change in the catalytic domain, and in future, an analysis of the higher-order structure of the mutant protein may lead to the development of new drugs stimulating production of cGMP in vivo.

In conclusion, we have identified a gain-of-function mutation of Npr2 in a family with tall stature and macrodactyly. Our findings reconfirm a critical role for NPR2 in skeletal growth in both humans and mice, and may provide a potential target for

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**Table 2.** Primer sets for the human Npr2 gene.

| Exon | Forward | Reverse | Product size (bp) |
|------|---------|---------|-------------------|
| 1–1  | CTGTAGGCCAGACGACGCC | AACCAGAGCCACACGAC | 441 |
| 1–2  | GACCCCCAGCCTGTTAG  | AGCCTTAGGAGCAACAGCACG | 454 |
| 2    | GGCAACTCCAGTGTCC | ACCATCTGCTGTTCC | 397 |
| 3    | AGATTGCCCTGCTGCAG | AGAGACCCCTCCCACAG | 253 |
| 4, 5 | ACCCGAGAGGGGAAAG | AGCTGACCCCTGAAAGGATG | 593 |
| 6, 7 | GAAACAGCTGGGGTCTGG | CTCTAGCCAGAGTTTGTG | 581 |
| 8, 9 | ACCCTGCCAGGCTGGCTG | GAAGGAGATGAGCAGGATG | 484 |
| 10, 11 | CCACTGGCTGCTAGCATTTCC | CAGTATAACCTTTGGCTCTTCC | 540 |
| 12   | ATGTCGAGGCTAGCTGCTG | CCAACATCAAAGACAGGTGAAG | 196 |
| 13, 14 | AGTACCCAGGCTCCTCCTC | TCTCTGAGGCCATCAAGAG | 640 |
| 15, 16 | TACCATGAGGCTCCTCCTTCC | GATGAAGGAGAGCCCAAC | 602 |
| 17, 18 | TGGACGGCATACACTTCTTCC | TGATGTTCCACAAGGGGCTTC | 516 |
| 19, 20 | GTGTCAGGGGCTCTCCCTC | GGATATTGAAAGAAAGAAAGG | 496 |
| 21, 22 | TGGGGACAGGCTGTATAC | GTGTTGCGAGCATGCGTG | 458 |

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**Table 3.** Primer sets for the human Npr3 gene.

| Exon | Forward | Reverse | Product size (bp) |
|------|---------|---------|-------------------|
| 1–1  | AAGAGTGGGGAGGAAAGG | ACTGGCGCTGCTGCATAC | 512 |
| 1–2  | CTTGATCTGGTGGACGG | CTGAGAGGGTGCAGGATG | 513 |
| 2    | TGCTAGCTGTCCCTCATGCTC | CACACCCCTCAGGAGATC | 599 |
| 3    | GAATTGGCTTCTTTAGTGAG | AATTGCTGAAAGGGGCAAC | 318 |
| 4    | CCGCCAGATGACGCTTTTGG | CAGCTGCAGAGAAATAC | 272 |
| 5    | TTTTGCTGGCAGAGCAGG | CAAGTACAGGGATGAGGATG | 225 |
| 6    | GAAATGCTGTGAGGACTTGG | CCACTACATCTGGAGGATGT | 276 |
| 7    | CCTCCAGGAGAATCAGG | CTGGTGAAGAAGGAGATG | 293 |
| 8    | AAGAGAACCATGATTTTGTAGC | CATTGTAGCTCTTACACGG | 246 |

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Notes:

- **doi:** 10.1371/journal.pone.0042180.t002
- **doi:** 10.1371/journal.pone.0042180.t003
prevention and treatment of diseases caused by impaired production of cGMP.

Materials and Methods

Mutation Analysis

The mutation analysis was approved by the ethics committee at Osaka University Graduate School of Medicine, and written informed consent was obtained from the proband and family members for the analysis of the natriuretic peptide precursor C (Nppc), Npr2, Npr3, and Fgfr3 genes.

All the exons of the 4 genes were amplified using specific primers flanking the intron-exon boundaries according to published human Nppc, Npr2, Npr3, and Fgfr3 genomic DNA sequences (UCSC genome browser: uc002vsl.1 at chromosome 2, 232498379–232499203; uc003zyd.1 at chromosome 9, 35782406–35799728; uc003jhv.2 at chromosome 5, 32711665–32787252; uc003gds.2 at chromosome 4, 1764337–1780396, respectively). Two primers for the Nppc gene, 14 primers for the Npr2 gene, 9 primers for the Npr3 gene, and 18 primers for the Fgfr3 gene were designed using the software Primer3Plus and synthesized to cover the genomic sequence from the start codon to the termination codon. The sequences of the primers are provided in Tables 1, 2, 3, and 4. Polymerase chain reaction (PCR) products were sequenced using a Big Dye terminator cycle sequencing kit (version 3.1; Applied Biosystems) and an ABI 3130 automated sequencer (Applied Biosystems).

Measurement of Serum Amino-Terminal proCNP Concentrations

Blood samples of III-1, II-5, I-2, and I-3 were centrifuged as soon as collected (15 min at 2,000 ×g, 4°C). The serum was stored

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### Table 4. Primer sets for the human Fgfr3 gene.

| Exon | Primer sequence (5′→3′) | Product size (bp) |
|------|-------------------------|-------------------|
| 1    | CGAGGGGGGGGCTGCCCTGGCCG | 339               |
| 2    | AGGGTCCGGAGCGCAAGAGG    | 350               |
| 3    | GTTCTGAACCCGGCGCGG      | 425               |
| 4    | ATCTGGAGGGGGACCTCTGGG   | 222               |
| 5    | GTTCAAGAGGGCCCTCTGTC    | 290               |
| 6, 7 | TCTGGCTCCGGTCATCTAC    | 554               |
| 8    | TCTCCCAACATCCTGTCCT     | 277               |
| 9    | AGGCGGCTGCTGGCGCTGC     | 228               |
| 10   | CAGGCCAACGGCTCAAGGCCG   | 271               |
| 11   | CTGTACTTCCACGGGCTCGC    | 264               |
| 12   | GAGTGGGAGGGGCGTCTGGG    | 211               |
| 13   | GTCAGAGAGGAGGCGTGGGGGC  | 240               |
| 14   | CTGGAAGCGCCGCTGGAGAGG   | 286               |
| 15   | GATGAGAGGCTTCAGCCTCT    | 217               |
| 16   | TCTGTCGCCAGTGGGAGCCTGGG | 184               |
| 17   | CGAGCGGAGGCTGGGGCCAATCC | 314               |
| 18   | GAAGCGGAGGCTGGGGGAC     | 180               |
| 19   | GCAAGGGGGCTGGGCGAC      | 254               |

doi:10.1371/journal.pone.0042180.t004

### Table 5. Primer sets used for the analyses of transgenic mice.

| Name | Forward | Reverse | Product size (bp) |
|------|---------|---------|-------------------|
| SV40SD/SA -HA | CTAGGGCTGTACGGAAGTGTAC | GTAATCTGGAAACATCGATGGTA | 154 |
| Npr2–SV40 poly(A) | TGGGAATGAAAGGGAAAGG | TCACGCATTCTGAGTTGGTTT | 149 |
| Sox9 | ACCGCGATCTGCAACGCCGGA | GCCTGATCTGTAATCGGG | 182 |
| Npr2 | TTTGGGCGCAAGCAT | GAGGTTGCTCAATATGTG | 164 |
| Gapdh | CCCGCTGACAAACATTGGAAG | ATGGCAAAATCTCCTCATTG | 104 |

doi:10.1371/journal.pone.0042180.t005

SV40SD/SA-HA and Npr2-SV40 poly(A) were used to amplify the fragment derived from the transgene.
at −80°C prior to use. NT-proCNP was assayed using an enzyme immunoassay (BIOMEDICA) according to the instructions provided. As a control, samples from 10 healthy adults (5 males and 5 females) were also measured.

Construction of Expression Plasmids
The pcDNA3.1(+) /hemagglutinin (HA)-tagged human NPR2 wild-type vector (HA-WT) was a gift from Dr. Yoshihiro Ogawa (Tokyo Medical and Dental University, Japan) [21]. The construct encoding the mutant p.Val883Met, pcDNA3.1(+) /HA-human NPR2 Val883Met (HA-Val883Met), was generated by PCR-based mutagenesis using HA-WT as the template, and primers containing the nucleotide change. All the vector constructs were verified by bidirectional DNA sequencing.

Cell Culture and Transfection
HEK293A cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Nacalai tesque) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acid, and 2 mM L-glutamine at 37°C with 5% CO2. HEK293A cells were plated at a density of 1×10⁶ cells/12-well plate and cultured one day so as to reach confluence. Transfection was performed using the liposomal transfection reagent FuGENE6 (Reagent : DNA = 3 μl : 0.5 μg, Roche), according to the manufacturer’s instructions. The cells were used for the experiments 48 hours (h) after transfection.

Immunoblot Analysis
The transfected cells were scraped into lysis buffer, sonicated for 2–3 sec and centrifuged to harvest the supernatant as the whole-cell extract. Five micrograms of protein was fractionated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted to a nitrocellulose membrane. A mouse monoclonal antibody against HA-tag (6E2, 1:1000; Cell Signaling Technology) was used as the primary antibody. The signals were detected using SuperSignal West Dura Extended Duration Substrate (Thermo SCIENTIFIC) with a horseradish peroxidase-linked sheep anti-mouse IgG antibody (1:10,000; Promega). An internal control, β-actin in each sample was detected with a monoclonal anti-β-actin antibody (1:4000; Sigma).

Assay for cGMP of Transfected Cells and Plasma Samples
Transfected cells were serum-starved for 24 h before the cGMP assay and then incubated at 37°C with 5% CO2 in DMEM containing 0.5 mM IBMX (3-isobutyl-1-methylxanthine) (Wako) for 10 min. The cells were next treated with 1×10⁻⁵–1×10⁻⁶ M CNP-22 (Biochem) or vehicle (water) and incubated for another 10 min. The reaction was terminated with 300 μl of 0.1 M HCl, and the cGMP concentration was measured by a competitive enzyme immunoassay (Cayman Chemical). Results are presented as the mean ± SD. Student’s t test was used for statistical analyses.

Plasma cGMP concentrations of III-1, II-5, I-2, I-3, and control females were also measured. As a control, samples from 10 healthy adults (5 males and 5 females) were also measured. As a control, samples from 10 healthy adults (5 males and 5 females) were also measured.

A soft X-ray analysis of the skeleton was performed using SOFTEX M-60 (30 kV, 3 mA, 80 sec, Softex), and faxitron X-ray (35 kV, 120 sec, Faxitron x-ray Corp).

Histological Analysis
Femurs were harvested, fixed in 4% paraformaldehyde, and decalcified in EDTA solution. Sections were cut from paraffin-embedded specimens of knee-joint, and stained with hematoxylin-eosin (H&E).
Acknowledgments

We thank Prof. Yoshihiro Ogawa for providing the expression vector for the human NPR2. We are grateful to Dr. Gen Nishimura for radiographic diagnosis.

Author Contributions

Conceived and designed the experiments: KM NN MF YO HI T. Kitaoka T. Kubota HH CH NS NT HY TM KO. Performed the experiments: KM KO TM NN T. Kitaoka MF HI T. Kubota NT. Analyzed the data: KO NN MF YO HI T. Kitaoka NS. Contributed reagents/materials/analysis tools: KM NN MF YO HI T. Kitaoka T. Kubota HH CH NS NT HY TM KO. Wrote the paper: KM KO TM NN.

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