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Intact Kinase Homology Domain of Natriuretic Peptide Receptor-B Is Essential for Skeletal Development

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Context: Natriuretic peptide receptor-B (NPR-B, GC-B in rodents; gene name *NPR2*) is a guanylyl cyclase-coupled receptor that mediates the effect of C-type natriuretic peptide. Homozygous mutations in human NPR-B cause acromesomelic dysplasia, type Maroteaux (OMIM 602875), an autosomal recessive skeletal dysplasia. NPR-B has an intracellular kinase homology domain (KHD), which has no kinase activity, and its functional significance *in vivo* is currently unknown.

Objective: We examined the functional significance of a novel NPR-B KHD mutation in humans.

Patients and Methods: A 28-yr-old Japanese male presented with marked short stature (118.5 cm, −9.3 SD). His limbs showed marked shortening in the middle and distal segments. His parents had relatively short stature with height z-scores of −2.75 and −0.98 (his father and mother, respectively). Direct sequencing of coding region of the *NPR2* gene of the family was performed. The mutant receptor activity was investigated by saturation binding assay and cGMP measurement. Additionally, interaction between the mutant and wild type allele was investigated by the titration experiments.

Results: We identified a novel missense mutation L658F in KHD of NPR-B in homozygous and heterozygous states in the patient and his parents, respectively. The mutation conferred normal binding affinity for C-type natriuretic peptide but no discernible ligand-induced cGMP production. Furthermore, L658F mutant impaired wild-type NPR-B-mediated cGMP production in a dose-dependent manner, suggesting that short stature found in L658F heterozygote can be caused by its dominant-negative effect.

Conclusions: This study provides the first evidence that intact KHD of NPR-B is essential for skeletal development. (***J Clin Endocrinol Metab* 92: 4009–4014, 2007**)

NATRIURETIC PEPTIDE RECEPTOR (NPR)-B [guanylyl cyclase (GC)-B, GC-B in rodents; gene name *NPR2*] is a receptor for C-type natriuretic peptide (CNP) that acts locally as a paracrine and/or autocrine regulator in a wide variety of tissues (1). Using the rodent models, we and others have demonstrated that the CNP/NPR-B pathway plays an important role in the regulation of bone formation *in vivo* (2–11). Recently Bartels *et al.* (12) reported that homozygous mutations in human NPR-B cause acromesomelic dysplasia, type Maroteaux (AMDM; OMIM 602875), a rare autosomal recessive skeletal dysplasia, indicating that NPR-B is also involved in human skeletal growth. This contrasts sharply with the role of NPR-A (GC-A; *NPR1*) that mediates two cardiac natriuretic peptides (atrial natriuretic peptide and brain natriuretic peptide); it is involved in the regulation of cardiovascular homeostasis (13).

Both NPRs (NPR-A and NPR-B) consist of an extracellular ligand binding domain, a single hydrophobic transmembrane region, an intracellular kinase homology domain (KHD), and carboxyl-terminal GC domain (1, 13). The KHD of NPRs is highly conserved in structure among species and is unique in that it has no kinase activity. However, the physiological role of KHD *in vivo* is poorly understood.

Here we report a novel homozygous missense mutation in KHD of NPR-B (L658F) in a Japanese patient with AMDM. Using the *in vitro* functional assay, we demonstrated that the mutation confers normal ligand binding affinity but impaired ligand-induced GC activity. This study represents the first demonstration that KHD of NPR-B is essential for bone development *in vivo*.

Patients and Methods

This study was conducted with informed consent from the patient and his parents and approved by the institutional review boards at Keio University School of Medicine, Saitama Children's Medical Center, and Medical Research Institute, Tokyo Medical and Dental University.

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Abbreviations: AMDM, Acromesomelic dysplasia, type Maroteaux; Bmax, maximum binding; CNP, C-type natriuretic peptide; GC, guanylyl cyclase; HA, hemagglutinin; Kd, dissociation constant; KHD, kinase homology domain; NPR, natriuretic peptide receptor; WT, wild type.

Case report

The patient is the second child of Japanese parents who are first cousins. He was born after vacuum extraction due to a nuchal cord at 40 wk of gestation. His birth weight was 3600 g (+0.92 SD), length 51 cm (+0.53 SD), and occipital-frontal circumference was 36 cm (+1.7 SD). He was initially given a diagnosis of achondroplasia at 3 months of age because of the short limb and relative macrocephaly. At the age of 28 yr, he was referred to Saitama Children's Medical Center for further examination. At the initial visit, his height was 118.5 cm (−9.3 SD), his weight was 34.5 kg (−2.7 SD), and his head circumference was 57.1 cm (+0.92 SD). He had a long face, hypertelorism, and a slightly flattened midface. His fingers were extremely short and broad, his first toes were relatively large, and the skin of his fingers was redundant. His limbs showed marked shortening in the middle and distal segments (Fig. 1, A–C). A skeletal survey revealed disproportionate mesomelic shortening of the arms with bowing of the radius, shortening of the phalanges and metacarpal bones, and mild platyspondyly and mild interpediculate narrowing of the vertebral column (Fig. 1, D and E). His intelligence was normal. Based on these observations, we concluded that he has AMDM rather than achondroplasia. He had been well except for the marked short stature until the age of 27 yr, when he was diagnosed as having abdominal aortic pseudoaneurysm by computed tomography scanning and was surgically treated with a stent graft. Six months after the operation, he had recurrence of pseudoaneurysm on the right common iliac artery (Fig. 1F) and underwent covered stent. There were no other AMDM patients in his family (Fig. 2A). His father was 155 cm (−2.75 SD) and his mother was 153 cm (−0.98 SD) in height. His elder brother was stillborn due to a nuchal cord. His elder sister was healthy.

Mutation analysis

We screened the patient and his parents for *NPR2* mutations. Genomic DNA was extracted from their peripheral blood using standard techniques. Bidirectional direct sequencing of *NPR2* using BigDye terminator cycle sequencing kit (version 1.1; Applied Biosystems, Foster City, CA) was analyzed with ABI 3130x automated sequencer (Applied Biosystems). The primers were designed to amplify all exons and exon-intron boundaries according to the published *NPR2* genomic DNA sequences (Ensembl Genome Browser accession no. ENSG00000159899). The primer sequences and PCR conditions are available upon request.

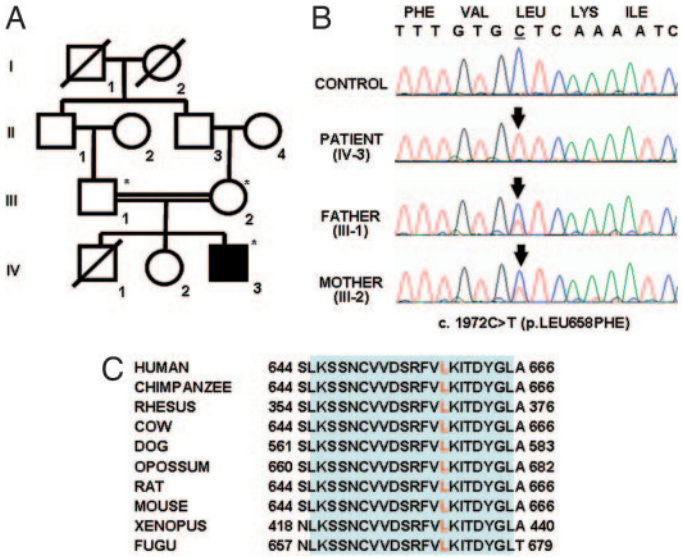

FIG. 2. Identification of *NPR2* mutation. A, Pedigree of the family of patient IV-3. *, Examined personally. B, Homozygous and heterozygous missense mutations (c.1972C>T) were detected in *NPR2* in the patient and his parents, respectively. c., cDNA, p., protein. C, Amino acid alignment of NPR-B among species. Leucine at codon 658 is in the highly conserved region (*highlighted*) in KHD.

Expression constructs and site-directed mutagenesis

The full-length cDNA clone of human NPR-B (GenBank accession no. BC096343; American Type Culture Collection, Manassas, VA) was subjected to PCR using adapter primers to introduce *Hin*dIII and *Xho*I sites into 5′ of the start codon and 3′ of the stop codon, respectively. The 3.1-kb insert was isolated from PCR product by *Hin*dIII and *Xho*I digestion and ligated into the pcDNA3.1 expression vector to generate pcDNA3.1/NPR-B wild-type (WT). To generate expression vectors with the hemagglutinin (HA) epitope immediately 3′ of the expected cleavage site of

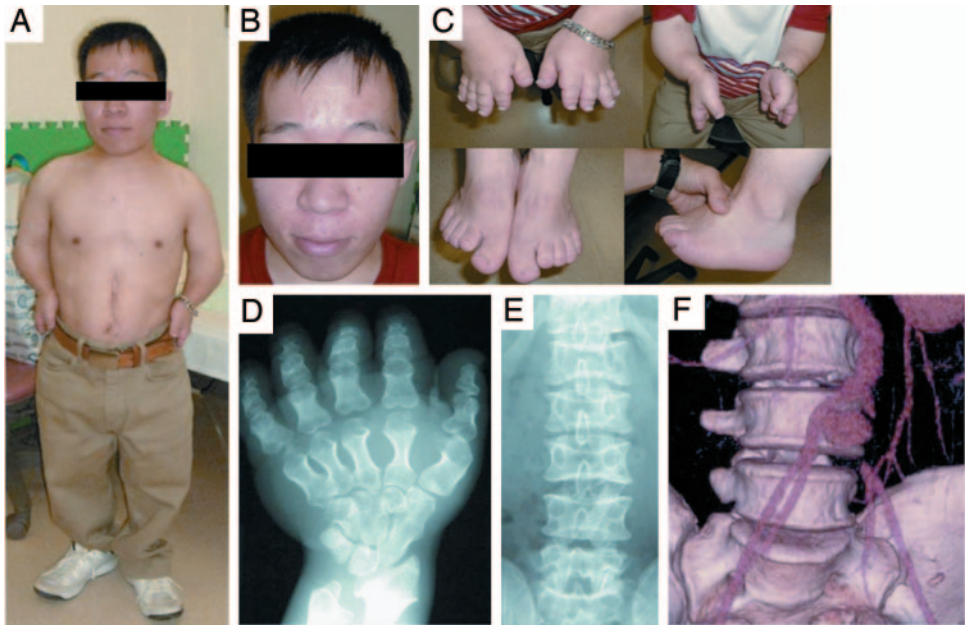

FIG. 1. Clinical features of the patient. A, Front full-length portrait. B, Face photograph. C, Hands and feet photographs. D, Hand radiograph. E, Spine radiograph. Note mild platyspondyly and interpediculate narrowing as revealed by the reduction in the L1-L5 heights and interpediculate distance, respectively. F, Three-dimensional computed tomography angiography revealed the pseudoaneurysm on right common iliac artery.

the signal sequence (~nucleotide +66), *Sac*II and *Hpa*I sites were created by site-directed mutagenesis at nucleotides +55 to +60 and +67 to +72, respectively. Linkers encoding the HA epitope were inserted between the *Sac*II and *Hpa*I sites to generate the HA-tagged wild-type clone; pcDNA3.1/HA-NPR-B wild-type (HA-WT). The mutant clone c.1972C>T (p.L658F) was generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) from WT and HA-WT as pcDNA3.1/NPR-B L658F (L658F) and pcDNA3.1/HA-NPR-B L658F (HA-L658F), respectively (Fig. 3A). All the expression vector constructs were verified by DNA sequencing.

Cell culture and transfection

COS-7 cells were seeded to 0.5–1 $\times$ 10^5 cells/cm^2 in 12- or 24-well plates in DMEM with 10% fetal bovine serum. The cells were transfected with pcDNA3.1 (mock), HA-WT, and HA-L658F alone or a combination of HA-WT and L658F in the titration experiments with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In the titration experiments, COS-7 cells were cotransfected with differing ratios of HA-WT and L658F DNAs with the amount of HA-WT DNA constant. The total amount of DNAs transfected was kept constant using the mock. The cells were used for the experiment 48 h after transfection.

Immunoblotting analysis

The transfected cells in 12-well plates were scraped into 150 μl/well of the lysis buffer [20 mM Tris (pH 7.4), 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1% Nonidet-P, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4], sonicated for 1 sec, and centrifuged at 17,000 $\times$ *g* for 15 min. The supernatant was fractionated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and blotted to the polyvinylidene defluoride transfer membrane (PerkinElmer, Waltham, MA). The HA-tagged NPR-B was detected by HA-tag (6E2) mouse monoclonal antibody (1:1000; Cell Signaling Tech-

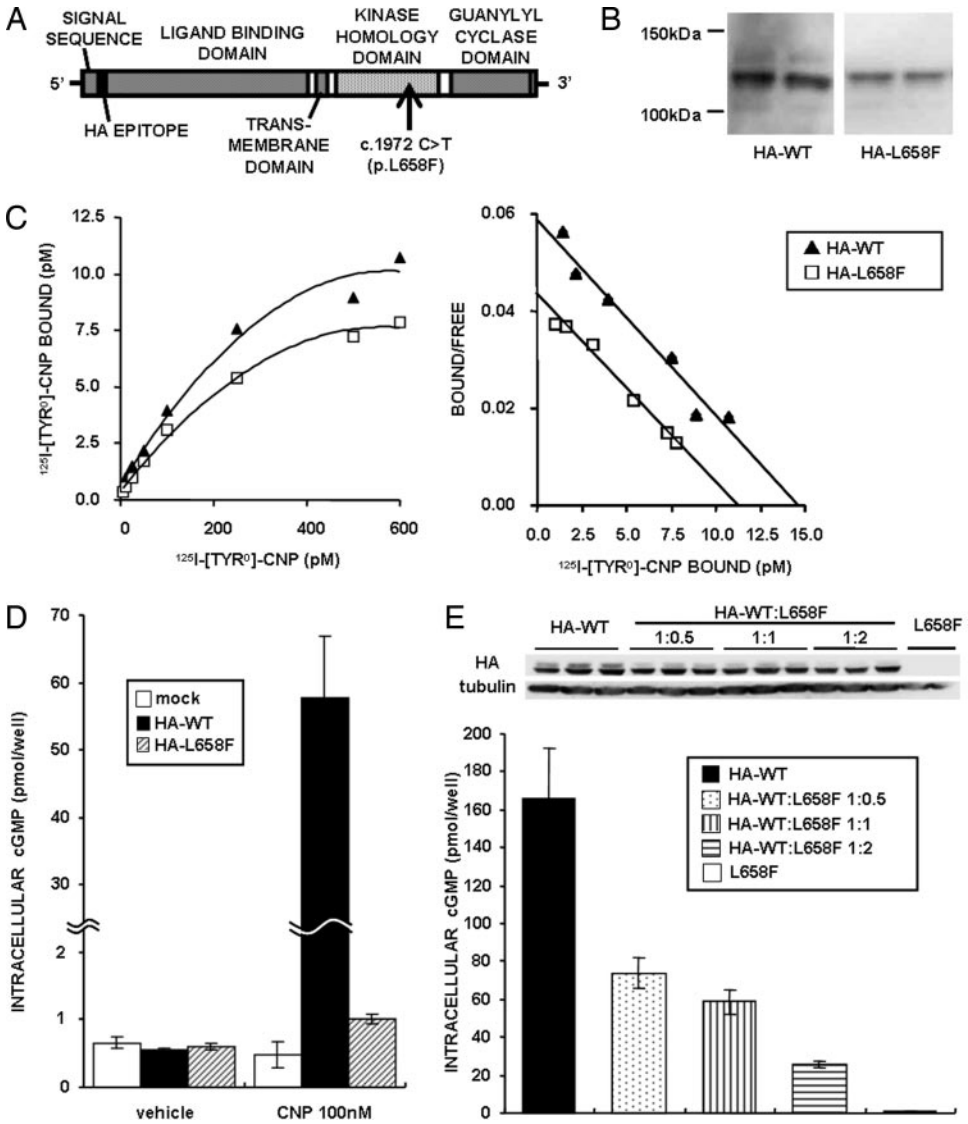

FIG. 3. *In vitro* functional assay. A, Schematic representation of *NPR2* cDNA with the HA epitope. The *upward arrow* denotes the nucleotide mutated by site-directed mutagenesis. B, NPR-B protein expression in COS-7 cells transfected with HA-WT or HA-L658F. Representative Western blots with anti-HA antibody are shown. C, CNP binding to NPR-B. *Left panel*, Binding of ^{125}I-[Tyr^0]-CNP to COS-7 cells expressing HA-WT or HA-L658F examined by saturation binding analysis. *Right panel*, Scatchard analysis. Linear regression curves are shown by *solid lines*. D, cGMP measurement. E, Dose-dependent effect of L658F on HA-WT. The ratios of HA-WT and L658F are those of transfected DNA concentrations. COS-7 cells were transfected with the constant amount of HA-WT. The total amount of DNAs transfected was kept constant in all transfections using mock. *Upper panel*, Representative Western blots with anti-HA and antitubulin are shown. Note HA-WT protein levels are roughly constant irrespective of the DNA concentrations of L658F mutant. *Lower panel*, cGMP production.

nology, Beverly, MA) using ECL plus detection system (GE Healthcare Bio-Science, Piscataway, NJ) with ECL antimouse IgG, horseradish peroxidase-linked sheep antibody (1:1000; GE Healthcare Bio-Science). As an internal control for the titration experiments, α-tubulin was detected by monoclonal anti-α-tubulin antibody (1:4000; Sigma-Aldrich, St. Louis, MO) and antimouse IgG antibody (1:10,000).

Saturation binding assay

Saturation binding assay with the intact cells were carried out as described with slight modification (14, 15). The transfected intact cells in 24-well plates were washed once with 200 μl of DMEM containing 0.1% BSA. Cells were then incubated at 37 C for 1 h with same medium with ^{125}I-[Tyr^{0}]-CNP (Peninsula Laboratories, San Carlos, CA) at various concentrations with or without 0.5 μM nonradioactive CNP (Bachem, Bubendorf, Switzerland) to define nonspecific binding. The cells were washed four times with 200 μl PBS containing 0.1% BSA and solubilized with 1 ml 0.5 N NaOH. Radioactivity of the cell lysate was counted.

cGMP assay

The transfected cells were serum starved for 12 h before cGMP assay and then incubated at 37 C in DMEM containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, for 10 min. The cells were treated with CNP (100 nM) or vehicle (PBS containing 0.1% BSA) and incubated for another 10 min. The stimulations were stopped with 0.1 M HCl, and cGMP was measured by the competitive enzyme immunoassay according to the supplier's instruction manual (cGMP enzyme immunoassay kit; Cayman Chemical, Ann Arbor, MI).

Statistics

Data were expressed as the mean ± SE. Dissociation constant (Kd) was determined using the inverse of the slope of the linear regression curves obtained by the Scatchard plot. Maximum binding (Bmax) was determined by extrapolation of the linear regression curves to the abscissa.

Results

Identification of NPR2 mutation

We identified a novel C>T missense mutation at the nucleotide position +1972 (c.1972C>T) that creates a leucine>phenylalanine substitution (p.L658F) in homozygous and heterozygous states in the patient and his parents, respectively (Fig. 2B). This variant was not reported as a single nucleotide polymorphism in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/) databases and not found in 100 alleles from Japanese healthy controls (data not shown). L658 is located in the highly conserved region in KHD of NPR-B or GC-B among species (Fig. 2C).

In vitro functional assay

To investigate the functional significance of the L658F mutation, we performed *in vitro* functional assay. COS-7 cells were transfected with HA-WT and mutant (HA-L658F) constructs, and the whole-cell extracts were resolved by SDS-PAGE and Western blotting. Western blot analysis confirmed that both HA-WT and HA-L658F are expressed in COS-7 cells with an approximate molecular size of 120 kDa (Fig. 3B).

In saturation binding assay, HA-WT and HA-L658F were capable of binding CNP with similar affinity (Fig. 3C, *left panel*). Scatchard plot analysis revealed a single class of high-affinity CNP binding sites for HA-WT and HA-L658F (HA-WT: Bmax 14.5 pM, Kd 246 pM; HA-L658F: Bmax 11.4 pM, Kd 266 pM) (Fig. 3C, *right panel*).

We also examined cGMP production in the cells transfected with HA-WT or HA-L658F. Treatment with CNP at a dose of 100 nM increased intracellular cGMP levels by more than 100-fold in HA-WT-transfected cells relative to mock-transfected cells (Fig. 3D). However, there was no significant difference between mock- and HA-L658F-transfected cells, when treated with CNP.

Dose-dependent effect of L658F on HA-WT cGMP production and expression was analyzed by the titration experiments. With HA-WT protein levels being roughly constant irrespective of the amount of the mutant, intracellular cGMP accumulation on CNP stimulation at a dose of 100 nM was abolished with increasing amounts of L658F (Fig. 3E).

Discussion

Here we identified a novel homozygous missense mutation in KHD of NPR-B in a Japanese patient with AMDM. We demonstrated that intact KHD of NPR-B is essential for skeletal development *in vivo*. The physiological role of KHD *in vivo* has been poorly understood so far; there have been no reports on animal models carrying KHD mutations. Moreover, the two missense mutations of KHD of NPR-B (Y708C and R776W) found in patients with AMDM were not functionally investigated (12). This study provides evidence that L658F, a missense mutation of KHD, markedly impairs ligand-induced cGMP production in a patient with AMDM. These observations indicate that KHD of NPR-B is essential for skeletal development *in vivo*.

It is likely that KHD is important for GC activation upon ligand binding. In this study, we found that L658F mutant binds CNP with normal affinity but does not lead to enzyme activation. Chinkers and Garbers (16) provided *in vitro* evidence that complete deletion of KHD from NPR-A results in constitutive activation of GC in the absence of the ligand and suggested that KHD of NPRs represses the enzymatic activity without ligand stimulation, which, upon ligand binding, is released and activated. *In vitro* studies with partial deletion or mutagenesis of KHD showed that putative ATP binding motif (^{519}LXGXXG^{525}) and phosphorylation sites (S513, T516, S518, S523, S526) are important for the receptor activation (17, 18). In this regard, Potter *et al.* (19) proposed a model for signal transduction; ATP binding and phosphorylation in KHD is required for sensitization of NPRs, which is followed by ligand-induced conformational change and enzyme activation. Because L658F mutant may not affect ATP binding and phosphorylation, we speculate that upon ligand binding, L658F mutant does not take properly the ligand-induced conformational change for enzyme activation. Further studies are required to elucidate the functional role of KHD in NPR-B signal transduction.

It is conceivable that NPR-B is involved in the regulation of endochondral ossification in humans. The patient described herein had marked shortening of the long bones and vertebrae, but his head circumference was within the normal range, suggesting the impairment of endochondral ossification. The precise role of NPR-B in human endochondral

ossification is currently unknown because growth plate histology has not been available from the patients with AMDM. The vertebrate models, mostly rodent models, have been useful to understand the human skeletal biology (20). Lacking CNP or GC-B, for instance, results in impaired endochondral ossification and severe dwarfism in mice (2, 3), and a natural mutation in the GC domain of GC-B in mice causes dwarfism (4), whereas transgenic overexpression (5–7) or reduced clearance (8, 9) of the ligands causes skeletal overgrowth. Moreover, targeted deletion or natural mutation of cGMP-dependent protein kinase II, a downstream target of cGMP, leads to the impairment of endochondral ossification (10, 11). Importantly, overexpression of CNP in chondrocytes has rescued a mouse model of achondroplasia (7). The above observations with rodents support the concept that NPR-B is involved in the regulation of human endochondral ossification.

The data of this study also suggest that short stature found in his parents is caused by a dominant-negative effect of the mutant allele. They are heterozygous for L658F mutation and have relatively short stature with height z-scores of −2.75 and −0.98 (for his father and mother, respectively). This study demonstrates that L658F mutant impairs the wild-type NPR-B-mediated cGMP production in a dose-dependent manner. Because L658F mutant has the intact extracellular domain required for dimerization (21), it is likely that L658F mutant forms a heterodimer with the wild-type NPR-B, thereby interfering its signal transduction in a dominant-negative fashion. In this regard, two recent studies (12, 22) reported that heterozygous carriers of NPR-B are associated with short stature, and the authors discussed that the haploinsufficiency may be the cause. On the other hand, Tamura and colleagues (3, 17) demonstrated that an alternatively spliced isoform of murine GC-B named GC-B2, which lacks a 25-amino acid stretch in KHD, acts as a dominant-negative isoform by virtue of blocking homodimer formation of the full-length GC-B1. Furthermore, Langenickel *et al.* (23) reported that a truncated GC-B mutant lacking most of the cytoplasmic domain acts as a dominant-negative molecule *in vitro* and *in vivo*. These observations are also consistent with the hypothesis that the dominant-negative effect can be the cause of short stature in heterozygous carriers of L658F mutation.

The patient had abdominal aortic pseudoaneurysm at the age of 27 yr. Given that NPR-B is expressed in a broad array of tissues including chondrocytes, brain, lung, uterus, and blood vessel (1, 3), it is conceivable that abdominal aortic pseudoaneurysm is related with NPR-B expression in the vessel wall. Further case reports are needed to better characterize the phenotype outside the skeleton, the prognosis and genotype-phenotype correlation of NPR-B mutations.

In conclusion, we report a novel homozygous missense mutation in KHD of NPR-B in a Japanese patient with AMDM. This study provides the first evidence that intact KHD of NPR-B is essential for skeletal development, thereby leading to the better understanding of the molecular mechanisms underlying skeletal development as well as the natriuretic peptide receptor biology.

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