Identification and Functional Characterization of Two Novel *NPR2* Mutations in Japanese Patients With Short Stature

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Context: C-type natriuretic peptide-natriuretic peptide receptor B (NPR-B) signaling is critical for endochondral ossification, which is responsible for longitudinal growth in limbs and vertebrae. Biallelic *NPR2* mutations cause acromesomelic dysplasia, type Maroteaux, which is bone dysplasia characterized by severe short stature and short limbs. A monoallelic *NPR2* mutation has been suggested to mildly impair long bone growth.

Objective: The goal of this study was to identify and characterize *NPR2* mutations among Japanese patients with short stature.

Subjects and Methods: We enrolled 101 unrelated Japanese patients with short stature. *NPR2* and *NPPC* were sequenced, and the identified variants were characterized in vitro.

Results: In two subjects, we identified two novel heterozygous *NPR2* mutations (R110C and Q417E) causing a loss of C-type natriuretic peptide-dependent cGMP generation capacities and having dominant-negative effects. R110C was defective in trafficking from the endoplasmic reticulum to the Golgi apparatus. In contrast, Q417E showed clear cell surface expression.

Conclusions: We identified heterozygous *NPR2* mutations in 2% of Japanese patients with short stature. Our in vitro findings indicate that *NPR2* mutations have a dominant negative effect, and their dominant-negative mechanisms vary corresponding to the molecular pathogenesis of the mutations. **(*J Clin Endocrinol Metab* 99: E713–E718, 2014)**

C-type natriuretic peptide (CNP)-natriuretic peptide receptor B (NPR-B) signaling plays a critical role in endochondral ossification, which is responsible for longitudinal growth in limbs and vertebrae (1–3). *NPR2* encodes NPR-B, which transduces CNP signals. Knockout mice for *Nppc* (encoding CNP) or *Npr2* are severely dwarfed (4, 5). Biallelic loss-of-function *NPR2* mutations cause acromesomelic dysplasia, type Maroteaux (AMDM), which is characterized by severe short stature and short limbs (6–9). In contrast, gain-of-function *NPR2* mutations were identified in patients with tall stature and macrodactyly (10, 11). These studies showed that CNP-NPR-B signaling is essential in skeletal development in humans.

In the first report of biallelic *NPR2* mutations causing AMDM, height SD scores (SDSs) of the probands' parents who were heterozygous carriers were lower than the mean

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Abbreviations: AMDM, acromesomelic dysplasia, type Maroteaux; CNP, C-type natriuretic peptide; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; ISS, idiopathic short stature; NPR-B, natriuretic peptide receptor B; PNGase F, peptide-N-glycosidase F; SDS SD score; WT, wild type.

height SDS of population-matched standards (7). In a large pedigree including an AMDM proband, who carried a homozygous mutation (p.I364fs), the mean height SDS of the heterozygous carriers was −1.8 ± 1.1, whereas that of the noncarriers was −0.4 ± 0.8 (12). Based on these two studies, it is presumed that heterozygous *NPR2* mutations can mildly impair long bone growth. Recently heterozygous *NPR2* mutations were detected in a Brazilian cohort with idiopathic short stature (ISS) (13).

In this study, we conducted genetic analyses of these two genes in 101 Japanese patients with short stature of unknown etiology and performed functional analyses to elucidate the precise molecular mechanisms of the identified mutations.

Subjects and Methods

Details of subjects and methods are described in the Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Subjects

We enrolled 101 unrelated Japanese patients with short stature who were recruited by the Japan Growth Genome Consortium, a research network of Japanese pediatric endocrinologists (14).

Sequencing

NPPC and *NPR2* were sequenced. Variations were filtered with reference to the dbSNP database and the 1000 Genomes Project database. Detected mutations were analyzed in 100 Japanese control individuals and the family members of mutation-carrying patients.

Functional characterization of mutant NPR-B

The hemagglutinin (HA)-tagged wild-type (WT) human NPR-B construct (HA-WT-NPR-B) has been described previously (8). All variants and myc-tagged WT NPR-B construct were generated. CNP-dependent cGMP response of transiently transfected COS7 cells were measured by a competitive enzyme immunoassay. Total protein expression of HA-NPR-B was determined by Western blotting. Deglycosylation experiments were performed, and the effect of brefeldin A was evaluated. The subcellular localization of HA-NPR-B was observed under a confocal microscope using immunofluorescence experiments. Co-immunoprecipitation experiments were performed after cotransfection of myc-WT-NPR-B and each HA-NPR-B construct.

Results

Characteristics of the subjects

The characteristics of the 101 subjects are shown in Supplemental Table 1.

Sequencing

We identified three novel *NPR2* variations (c.328C>T, p.R110C; c.559G>A, p.V187I; and c.1249C>G, p.Q417E) in three subjects and no novel *NPPC* sequence variation (Figure 1A). These three *NPR2* variations were absent in the 100 con-

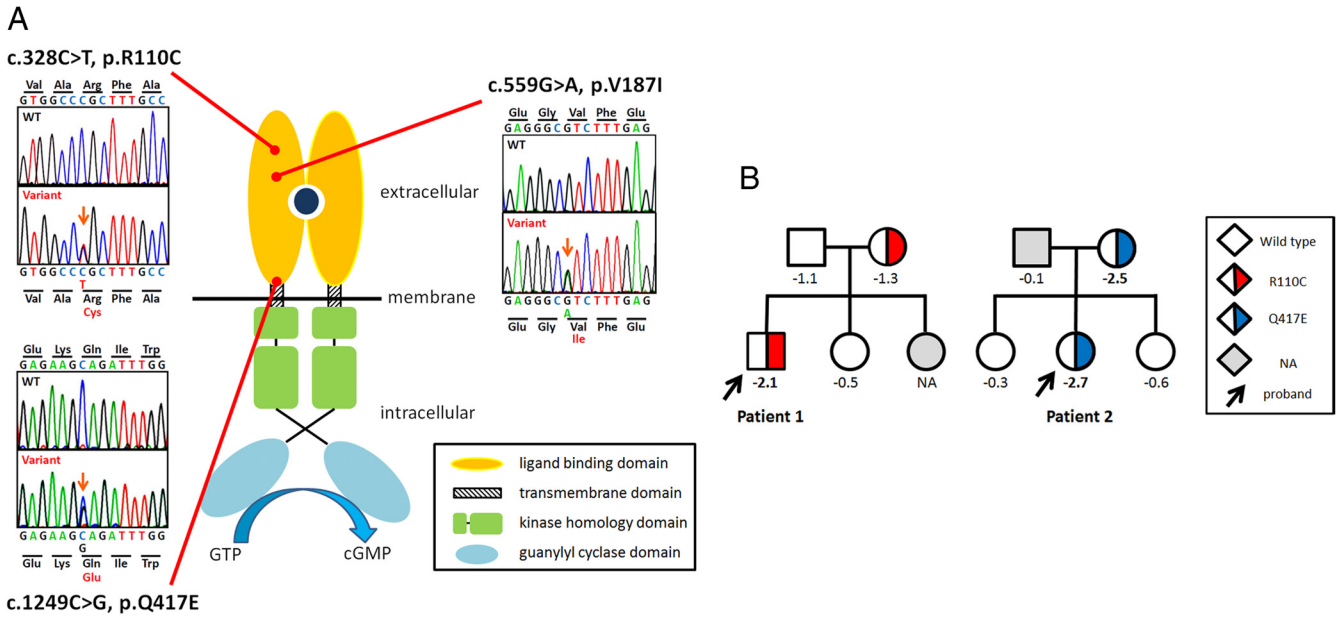

Figure 1. Identification of three *NPR2* sequence variations. A, A schematic diagram of NPR-B protein, which acts as a dimer and has an extracellular ligand binding domain, a transmembrane domain, a kinase homology domain, and a guanylyl cyclase domain. The blue closed circle is CNP. Three sequence variations are visualized by chromatograms. The three heterozygous substitution of cysteine in place of Arg110, isoleucine in place of Val187, and glutamic acid in place of Gln417 are indicated by arrows. The three sequence variations are located in the ligand binding domain. B, Pedigrees of patient 1 and patient 2 are shown. The values under the symbols show height SDS. NA, not available.

trol individuals. The three *NPR2* variants were located in the extracellular domain.

Functional characterization of NPR-B variants

To examine the pathogenicity of the three variations, we evaluated the CNP-dependent, cGMP-producing capacities. R110C showed a negligible cGMP response, and Q417E showed a markedly low response (Figure 2A). V187I had a comparable response with WT, indicating that the variant is functionally neutral.

We characterized HA-WT-NPR-B using Western blotting. HA-WT-NPR-B (total cell lysate, untreated) showed two bands (Figure 2B). Peptide-N-glycosidase F (PNGase F) treatment showed a single digested product band of smaller molecular size (Figure 2C), indicating that the two bands were derived from N-linked glycosylated protein. The lower band was sensitive to endoglycosidase H (Endo H), which digests the sites of N-glycosylation occurring in the endoplasmic reticulum (ER), whereas the upper band was digested by O-glycosidase, which digests the sites of O-glycosylation occurring in the Golgi apparatus. These results indicate that the lower band corresponded to N-glycosylated NPR-B produced in the ER, whereas the upper band corresponded to the mature NPR-B with N- and O-glycosylation produced in the Golgi apparatus. Treatment with brefeldin A, which inhibits protein transportation from the ER to the Golgi apparatus, resulted in the same immunoreactive pattern as that of O-glycosidase (Figure 2D). This result also indicates that the upper band was derived from the fully glycosylated NPR-B in the Golgi apparatus.

To clarify the mechanisms underlying the two loss-of-function mutants, we conducted a series of expression experiments in vitro. We performed Western blotting of the mutant HA-NPR-B. R110C showed only the lower band, whereas Q417E appeared as two bands (Figure 2E), indicating that R110C did not exist in the O-glycosylated form. We analyzed the subcellular localization of HA-NPR-B with an HA epitope in the extracellular domain. Under the nonpermeabilized condition, WT and Q417E displayed cell surface expression, whereas R110C did not (Figure 2F). Under the permeabilized condition, R110C was colocalized with an ER marker (Figure 2F).

We assessed possible dominant-negative effects of the two mutants. Coexpression of each mutant and WT led to a significant loss in the CNP-dependent cGMP response compared with that of the empty vector and WT, indicating a dominant-negative effect (Figure 2G).

To elucidate mechanisms causing the dominant-negative effects, we conducted further expression experiments. We performed Western blotting using cells coexpressing HA-WT-NPR-B and HA-R110C-NPR-B (Figure 2I). A decrease in the upper band (fully glycosylated receptor) and parallel an increase in the lower bands (N-glycosylated, not O-glycosylated) were observed in cells coexpressing the R110C mutant. To evaluate the interaction between WT and each mutant, we performed coimmunoprecipitation (Figure 2H). After precipitation of total cell lysates with an anti-HA antibody, immunoblotting of the precipitates with an antimyc antibody showed that both HA-R110C-NPRB and HA-Q417E-NPRB interacted with the myc-WT-NPR-B.

Family analyses and clinical phenotypes

The mutations (R110C and Q417E) were transmitted from their mothers (Figure 1B). Patient 1 showed normal serum IGF-I and normal GH responses by stimulation tests of arginine, L-DOPA, and growth hormone releasing hormone. After introduction of recombinant human GH treatment (50 μg/kg·d) for 2 years, the patient's height velocity slightly improved. However, the patient's adult height SDS was −2.8 (Supplemental Figure 1). Height SDS of the patient's mother was −1.3. Patient 2 also showed normal serum IGF-I level, and the patient's adult height SDS was −2.6. The patient's mother had short stature (height SDS, −2.5) (Supplemental Table 2).

Discussion

In the present study, we identified three *NPR2* variants (R110C, V187I, and Q417E) in a Japanese short stature cohort. The Arg110 residue is highly conserved among vertebrate species, whereas Val187 and Gln417 are conserved among terrestrial animals but not in fish (Supplemental Figure 2). Functional studies showed that R110C and Q417E lost the CNP-dependent cGMP generation capacities and had dominant-negative effects in different manners in vitro.

We identified heterozygous *NPR2* mutations in 2% (95% confidence interval 0%–7%) of Japanese patients with short stature. Assuming that the prevalence of AMDM is 1 in 2 000 000 by comparing the number of patients with AMDM with that with achondroplasia in the Little People of America, regardless of ethnicities, the frequency of heterozygous *NPR2* mutation carriers is calculated to be approximately 1 in 700 (12). Hypothesizing that the effect of a heterozygous *NPR2* mutation on height SDS is −1.8 according to the previous report, 2.6 in 100 subjects with short stature, which is defined as height SDS less than −2.0, are expected to be heterozygous for the *NPR2* mutation. This estimation is consistent with our observation. Recently *NPR2* mutations were identified in 6% of the Brazilian cohort with ISS (13). Further studies

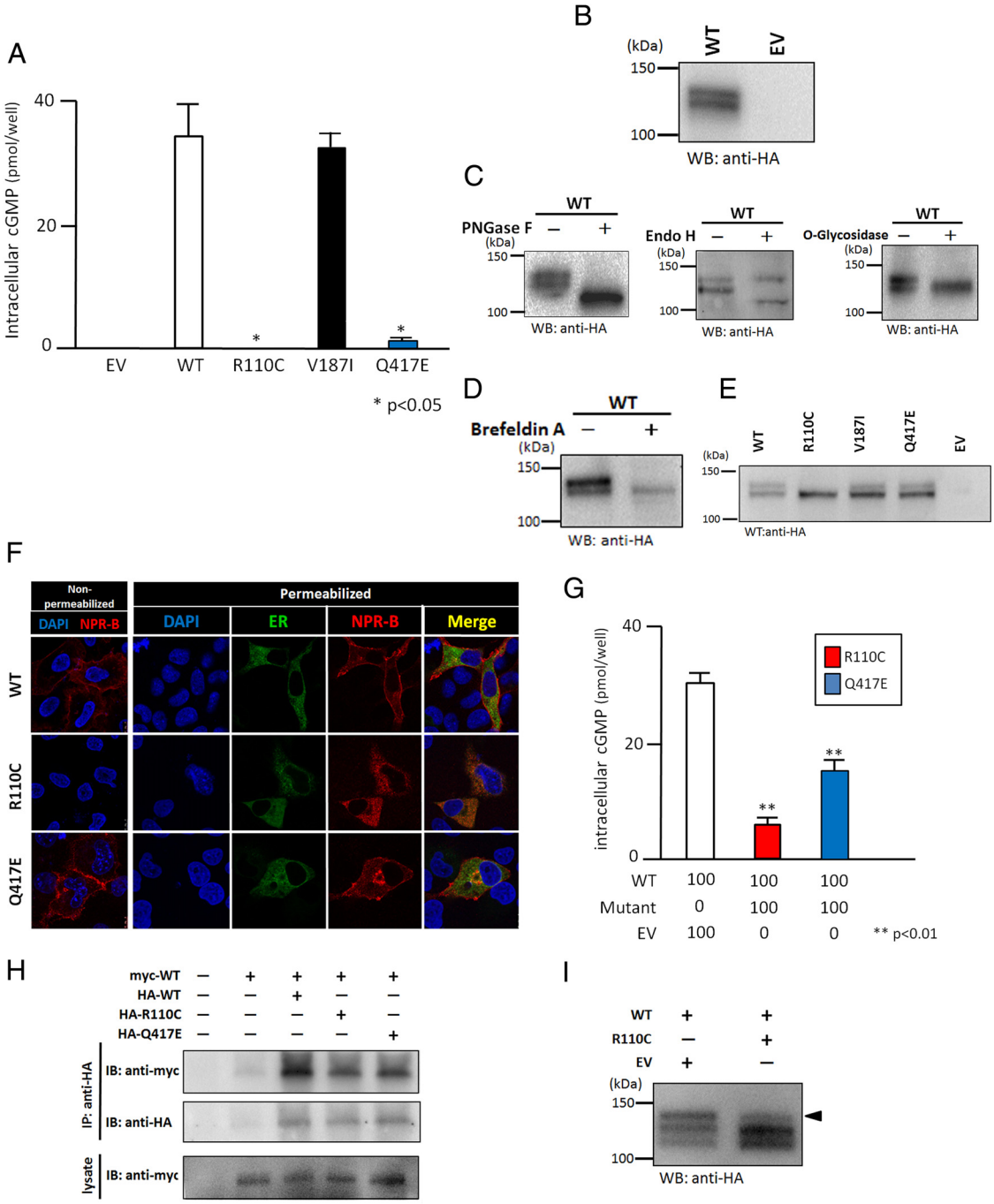

Figure 2. Functional characterization of the three NPR-B variants. A, CNP-stimulated cGMP accumulation in COS7 cells transiently transfected with empty vector (EV), wild-type (WT), and the three NPR-B variants (R110C, V187I, and Q417E) were evaluated. Data are presented as the mean ± SEM of triplicate samples and are representative of three independent experiments. B, Western blotting analysis of COS7 cells expressing HA-WT-NPR-B revealed two bands. C, Western blotting analysis of HA-WT-NPR-B, which was deglycosylated with PNGase F, Endo H, or O-glycosidase/neuraminidase is shown. Both bands were sensitive to PNGase F (left panel). The lower band was digested by Endo H (middle panel), whereas the upper band was sensitive to O-glycosidase/neuraminidase (right panel). D, Western blotting analysis of HA-WT-NPR-B under treatment with brefeldin A is shown. Only the lower band was detected. E, Western blotting analysis of HA-NPR-B (WT, R110C, and Q417E) shows that WT and Q417E revealed two bands, whereas R110C did as a single band. Migrations of molecular mass markers (in kilodaltons) are shown on the left of each panel in B–E. F, Subcellular localization analyses of HA-NPR-B proteins in COS7 cells under the nonpermeabilized or permeabilized condition are shown. WT (red) and Q417E (red) showed clear plasma membrane expression in nonpermeabilized cells, whereas R110C (red) colocalized with an ER (green) marker in permeabilized cells. G, CNP-stimulated cGMP accumulation in cells transfected with WT/R110C or WT/Q417E NPR-B is shown. H, Coimmunoprecipitation analysis of WT and the mutants is shown. Lysates from cells expressing myc-WT-NPR-B and HA-NPR-B (WT or mutants) were precipitated (IP) with an anti-HA antibody. The myc-WT-NPR-B alone and myc-WT-NPR-B being coexpressed with HA-WT-NPRB were used as negative and positive controls, respectively. Samples were assessed by Western blotting using anti-myc (top panel) and anti-HA antibodies (middle panel). As a control, the amount of myc-HA-NPR-B was verified in total cell lysates (bottom panel). The bands corresponding to HA-NPR-B (R110C and Q417E) were present in immunoprecipitates from cells coexpressing myc- and HA-WT-NPR-B (top panel). I, Western blotting analysis in cells coexpressing WT and R110C showed that the upper band (arrowhead) decreased and the lower bands increased.

are required to elucidate whether the frequencies of heterozygous mutation carriers in ISS differ, depending on ethnicities.

R110C showed defective trafficking from the ER to the Golgi apparatus. Subcellular localization studies have shown that 11 of 12 missense *NPR2* mutations, which were identified in AMDM patients, caused ER retention (15). Therefore, defective cellular trafficking from the ER to the plasma membrane is likely a major molecular mechanism of the missense *NPR2* mutations. Protein folding in the ER is monitored by ER quality control mechanisms, and misfolding proteins are retained and degraded in the ER by an ER-associated degradation pathway (16). We speculate that a change in conformation of the mutant NPR-B receptor promotes protein misfolding and defects in the normal intracellular trafficking from the ER to the Golgi apparatus.

In contrast to R110C, Q417E was expressed normally on the plasma membrane. We hypothesize that the pathogenesis of Q417E may be defective in ligand binding or receptor activation. Particularly, Gln417 is located in the junctional region between the ligand binding domain and the transmembrane domain. According to an investigation using an NPR-A crystal structure, counterclockwise rotation of juxtamembrane regions of a dimer is prerequisite for initiating transmembrane signaling by ligand binding (17). This rotation, which is transduced across the membrane, can reorient the intracellular domains and activate guanylyl cyclase. If the rotation of the NPR-A receptor is applied to the NPR-B receptor, a juxtamembrane region of NPR-B, including Gln417 might also play a critical role in initializing transmembrane signaling.

R110C and Q417E showed dominant-negative effects on the coexpressed WT receptor. Previously, four missense mutations (S76P, R263P, L658F, and R819C) were demonstrated to have dominant-negative effects (8, 13), but molecular mechanisms have not been elucidated. Our coexpression experiments indicated that R110C, which has defective trafficking from the ER to the Golgi apparatus, interacted with WT-NPR-B and relatively reduced the abundance of a fully glycosylated receptor. Some receptor mutants (eg, GnRH receptor, melanocortin 1 receptor, and α2-adrenergic receptor) were reported to have dominant-negative effects by heterodimerizing with and entrapping the WT receptor in the ER (18–20). A similar pathological mechanism can be true for the dominant-negative effect of R110C. Our coexpression experiments also showed that Q417E expressing on the plasma membrane interacted with WT-NPR-B. Q417E probably functions as a dominant-negative mutant, suppressing the activation and response of WT-NPR-B by forming an unproductive heterodimer, WT-NPR-B/Q417E.

In contrast, the I364fs mutant lacking the transmembrane domain was not thought to have a dominant-negative effect (ie, haploinsufficiency) (12). These findings necessitate the reinvestigation of other heterozygous *NPR2* mutations to discriminate whether the pathological effects (a dominant negative effect or haploinsufficiency) on WT-NPR-B in vitro significantly influence clinical phenotypes.

We provided observational data that were consistent with the hypothesis that a monoallelic *NPR2* mutation could cause short stature. However, we have not rigorously verified that hypothesis. It would be of interest to have genotype and phenotype information for the extra family member, as reported in the paper by Olney (12).

In summary, we identified two heterozygous loss-of-function *NPR2* mutations in a Japanese cohort with short stature. Both mutations had a dominant-negative effect, and their dominant-negative mechanisms varied corresponding to the molecular pathogenesis of *NPR2* mutations. Further studies involving other mutants should be conducted to clarify the pathological roles of different mutations in long bone growth.

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