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SHORT COMMUNICATION

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A novel activating mutation (C129S) in the calcium-sensing receptor gene in a Japanese family with autosomal dominant hypocalcemia

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Abstract Autosomal dominant hypocalcemia can be caused by activating mutations of the calcium-sensing receptor (*CaSR*) gene. We experienced two patients (proband and her daughter) with hypocalcemia caused by a missense mutation of the *CaSR* gene. The proband, aged 25, showed hypocalcemia and hypoparathyroidism from infancy. She had been diagnosed as having idiopathic hypoparathyroidism and had been treated with calcitriol. She gave birth to a female infant at age 24 years. Her daughter was found to have hypocalcemia (Ca, 6.6 mg/dl), without seizure or tetany, when she was 7 months old. DNA analysis of their *CaSR* genes showed a novel heterozygous mutation at codon 129 (TGC-to-AGC) with substitution of cysteine for serine (C129S). Familial examination revealed that this mutation had occurred de-novo in the proband. Wild-type and mutant (C129S) *CaSR* cDNA were transfected into HEK293 cells, and intracellular calcium concentrations were measured with a fluorescent calcium indicator. HEK cells transfected with the C129S mutant *CaSR* gene showed a larger increase in intracellular calcium concentration in response to the change in the extracellular calcium concentration than HEK cells transfected with the wild-type receptor. We conclude that the C129S mutation in the *CaSR* gene observed in these patients causes autosomal dominant hypocalcemia.

Key words Calcium-sensing receptor · Autosomal dominant hypocalcemia · Hypoparathyroidism · Missense mutation · Intracellular calcium

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Introduction

The calcium-sensing receptor (CaSR) is a member of the seven-transmembrane domain G-protein-coupled receptor superfamily. With mutations of the *CaSR* gene, hypercalcemia or hypocalcemia can occur (Brown et al. 1993; Garrett et al. 1995). Mutations in the *CaSR* gene that cause gain in the receptor function have been reported in patients with autosomal dominant hypocalcemia (Mancilla et al.1997; Lovlie et al. 1996; Pearce et al. 1996b; Okazaki et al. 1999) and in sporadic hypocalcemia (Baron et al. 1996; De Luka et al. 1997). Even when extracellular calcium concentration declines, *CaSR* with activating mutations inappropriately suppresses the secretion of parathyroid hormone, which increases serum calcium levels, resulting in hypocalcemia. In previous reports, mutations that cause autosomal dominant hypocalcemia have been detected in the extacellular domain of the proximal amino-terminal region or the transmembrane region of the receptor. We experienced familial hypocalcemia (proband and her daughter), and confirmed by DNA analysis that a novel mutation at codon129 of *CaSR* had occurred in the proband, and was transmitted to her daughter.

Patients and methods

The proband (case 1) was a 25-year-old woman. She had manifested hypocalcemia in infancy. From seven months of age, she had experienced convulsions and was diagnosed as having epilepsy. When she was 8 years old, she visited our hospital and showed hypocalcemia (Ca, 6.0 mg/dl) and hyperphosphatemia (inorganic[i]P, 10.2 mg/dl). Ellsworth-Howard test showed a normal response of urinary cyclic adenosine morophosphate (cAMP) excretion. Thus, she was diagnosed as having idiopathic hypoparathyroidism, and calcitriol therapy was started. When she was 19 years old, she had nephrocalcinosis and her serum creatinine level was increased, to 1.3 mg/dl. At the age of 24 years, she gave

birth to a female infant (case 2). The daughter was asymptomatic during early infancy. When she was 7 months old, she was found to have hypocalcemia (Ca, 6.6 mg/dl) and hyperphosphatemia (iP, 9.5 mg/dl). Her intact parathyroid hormone (PTH) level was below the normal range (<5 pg/ml). The urinary calcium/creatinine ratio in the proband and her daughter exceeded 0.21, when serum calcium concentrations were 7.0 mg/dl in case 1 and 8.4 mg/dl in case 2.

Because the disease was apparently transmitted in a dominant form, we performed DNA analysis of the *CaSR* gene after obtaining written informed consent from the patient for herself and her daughter. Lymphocytes were collected from heparinized peripheral blood samples and DNA was extracted with the Sepa Gene kit (Sanko Jyunyaku, Tokyo, Japan). Exon 2 to exon 7 of the CaSR gene, which codes the entire the *CaSR*, were amplified by the polymerase chain reaction (PCR) procedure (Pollak et al. 1993). The PCR products were analyzed with the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA), and the entire sequence of the coding region of the *CaSR* gene was determined. For expression experiments, wild-type *CaSR* cDNA was inserted into a pcDNA3 vector (pcDNA3CaSR) (Invitrogen, Carlbad, CA, USA), and a mutant expression plasmid was constructed by in-vitro site-directed mutagenesis (Gene Editor in vitro Site-Directed Mutagenesis System, Promega, Madison, WI, USA) to replace cysteine-129 to serine (pcDNA3CaSR-C129S). HEK293 cells were transfected with the wild-type or the mutant plasmid, and stable cell lines expressing receptors were selected by adding G418 to the culture medium. We measured the cytosolic calcium changes in single cells as described previously (Miyauchi et al. 2000). Briefly, HEK293 cells expressing either wild-type or mutant *CaSR* were cultured for 1 to 2 days on glass cover slips and loaded with 5 μM fura 2-AM (Dojindo Laboratories, Kumamoto, Japan), a fluorescent calcium indicator, for 1 h at 25°C in the bath solution (126 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM Na-hydroxyethylpiperazine ethanesulfonic acid [HEPES] (pH 7.4), and 0.5% bovine serum albumin). The measuring chamber was mounted on a microfluorometric system, and images were collected at wavelengths of 340 nm (F340) and 360 nm (F360). The F340/F360 ratio, constructed after background image subtraction, disclosed the intracellular Ca^{2+} ($[Ca^{2+}]_i$) localization. The average cell $[Ca^{2+}]_i$ values were calculated from the calibration curve as previously described (Miyauchi et al. 2000). High extracellular Ca^{2+} challenge was performed by replacing the medium with other media at various Ca^{2+} concentrations.

Results and discussion

DNA analysis revealed that the *CaSR* gene in both the proband and her daughter had a heterozygous mutation in exon 2 at codon 129 (TGC-to-AGC) (Fig. 1A) that caused a substitution of the amino acid cysteine to serine. This substitution created an *Alw*N1 restriction site. Examination

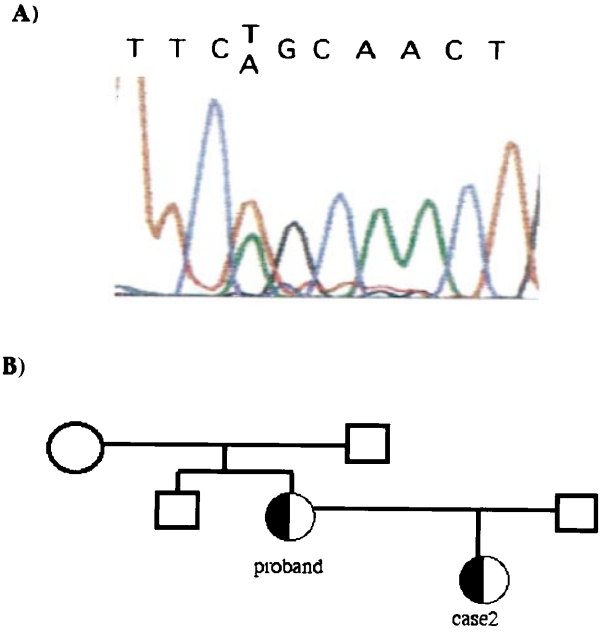

Fig. 1. A DNA analysis of the *CaSR* gene in case 1. Entire coding regions of the *CaSR* gene were amplified by polymerase chain reaction (PCR) from the genomic DNA of the patients, and the DNA sequence was determined by the direct sequence method. A heterozygous mutation at codon 129 (TGC-to-AGC) was revealed in case 1. The same mutation was seen in case 2. **B** Pedigree of the patients. Mutation of the *CaSR* gene was detected only in the affected members of the family (the proband and her daughter)

of the parents and the brother of the proband (Fig. 1B) with enzyme-based and direct sequence analysis revealed that this mutation had occurred in the proband de novo. We did not find this mutation in 100 alleles in 50 healthy volunteers without hypocalcemia. In addition, this nucleotide change did not exist in more than 100 normal subjects (Heath et al.1996). In the expression experiments, high extracellular Ca^{2+} (0.5 to 5 mM) caused a rapid and sustained increase of $[Ca^{2+}]_i$ in both HEK293 cells expressing C129S and wild-type *CaSR*. The net increase in $[Ca^{2+}]_i$ was dependent on the change in extracellular Ca^{2+}. The dose-response curve to extracellular Ca^{2+} in the HEK293 cells expressing C129S mutant *CaSR* was shifted to the left, with effective concentrations (EC) 50 values of approximately 0.8 mM, compared with the wild-type transfectant EC 50 value of approximately 2.2 mM) (Fig. 2). Thus, to our knowledge, this is the first report that the mutation at cysteine-129 of the *CaSR* gene (Fig. 3) causes autosomal dominant hypocalcemia.

Extracellular calcium concentration is regulated mainly by PTH secretion from the parathyroid gland. CaSR senses extracellular calcium concentration and regulates PTH synthesis. PTH secretion is inappropriately suppressed with lower extracellular calcium concentration by activating mutations of *CaSR*. Although, at present, it is still not clear whether CaSR exists as a dimer in the cell membrane or not (Bai et al. 1998), Watanabe et al. (1998) demonstrated that the co-expression of the wild-type and the activating mutant *CaSR* shifted the set-point of the extracellular calcium concentration to almost equivalent to that observed

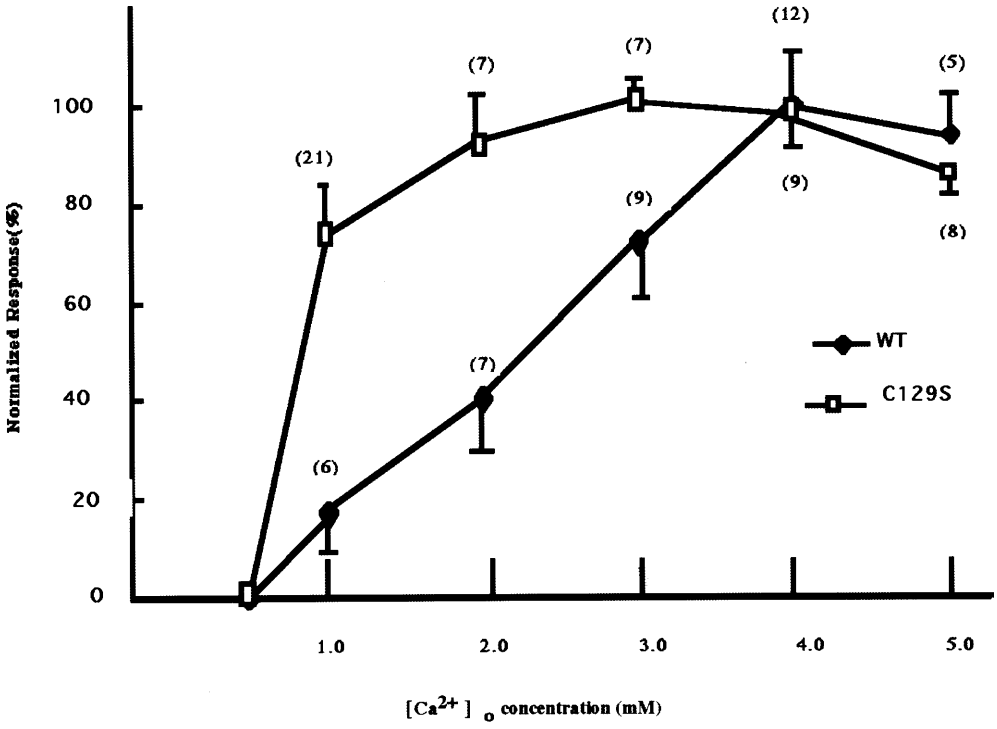

Fig. 2. Functional analysis of wild-type (*WT*) and mutant C129S *CaSR*. HEK293 cells expressing wild-type or mutant *CaSR* were grown on a slide glass, and the change in the intracellular calcium concentration was measured in a single cell. Values for results were expressed as means ± SEM. The number of experiments is indicated in *parentheses*

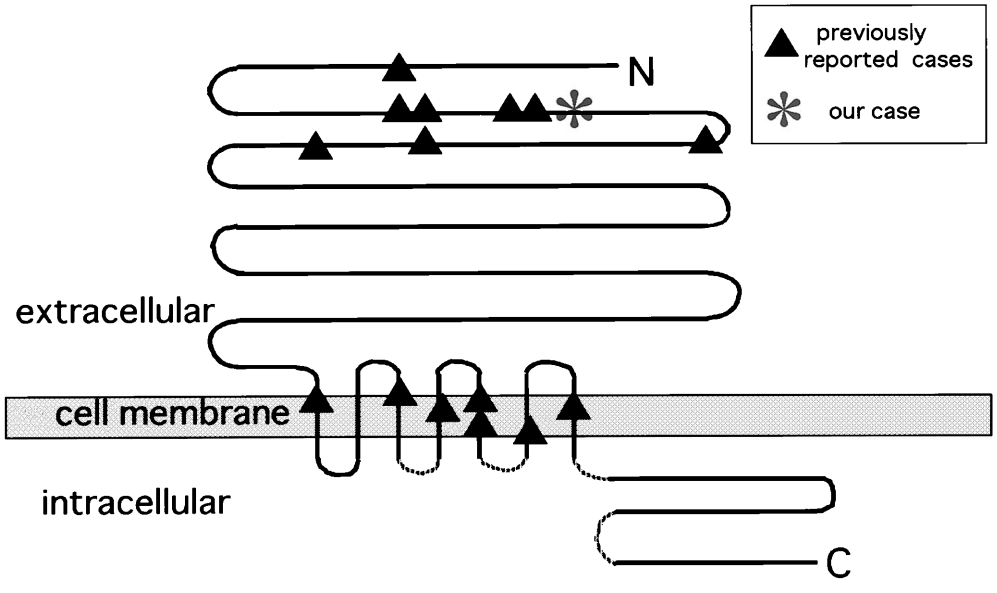

Fig. 3. Portions of activating mutations in *CaSR*. Activating mutations reported previously existed at the N-terminal in the extracellular domain or in the transmembrane domain. The mutation in our cases (probond and her daughter) exists at the N-terminal in the extracellular domain

with mutant receptor alone. Taken together, these findings indicate that the activating mutation of the *CaSR* gene in one allele causes the hypocalcemia inherited in autosomal dominant form.

The extracellular domain of *CaSR* does not contain the consensus sequences proposed for high-affinity Ca^{2+} binding sites, but a low-affinity Ca^{2+} binding site does exist in this domain (Brown et al.1993). The precise mechanism by which *CaSR* transmits the change in the extracellular calcium concentration is unknown, but the low-affinity Ca^{2+} binding site may play a significant role. Point mutations of glutamic acid-127 (Pollak et al.1994) and phenylalanine-128 (Pearce et al. 1996a) were previously reported to cause autosomal dominant hypocalcemia. Because the mutation we report here was located at codon 129, this region seems to be significant in regard to the binding to extracellular calcium. Previously, hypocalcemia was reported to be more severe in patients with mutations in the transmembrane domain than in those with mutations in the extracellular domain of the *CaSR* gene (Watanabe et al. 1998). Considering that the proband presented here apparently manifested more severe symptoms than her daughter, the severity of

autosomal dominant hypocalcemia seems to be determined not only by the type of mutation but also by other factors, such as the calcium nutritional status.

In conclusion, the novel point mutation at cysteine-129 in the extracellular domain of *CaSR* causes autosomal dominant hypocalcemia, and this result indicates that this amino acid plays an important role in binding to extracellular calcium ions.

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