

NOTE

## A Family of Autosomal Dominant Hypocalcemia with an Activating Mutation of Calcium-Sensing Receptor Gene

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**Abstract.** Autosomal dominant hypocalcemia (ADH) caused by activating mutations of calcium-sensing receptor (CaSR) is characterized by hypocalcemia with inappropriately low concentration of PTH and relative hypercalciuria. Active vitamin D treatment often leads to nephrolithiasis and renal impairment in patients with ADH. However, differential diagnosis between ADH and idiopathic hypoparathyroidism is sometimes very difficult. Here, we report a mutation of CaSR and its functional property found in three generations of a Japanese family. The proband developed seizures at 7 days of age. His mother and elder sister were discovered to have hypoparathyroidism by family survey, but his father was normocalcemic. His grandfather developed heart failure and was found to have hypoparathyroidism. All affected members had been treated with active vitamin D<sub>3</sub> and bilateral nephrolithiasis were detected in three of them. DNA sequencing revealed that all affected patients had a heterozygous mutation in CaSR gene that causes proline to leucine substitution at codon 221 (P221L). *In vitro* functional analysis of the mutant CaSR by measuring inositol 1,4,5-trisphosphate production in response to changes of extracellular Ca indicated that this mutation is an activating one and responsible for ADH in this family. Therefore, careful monitoring of urinary Ca excretion before and during treatment of PTH-deficient hypoparathyroidism is very important, and screening of CaSR mutation should be considered in patients with relative hypercalciuria or with a family history of hypocalcemia.

**Key words:** Autosomal dominant hypocalcemia, CaSR, Activating mutation

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**CALCIUM-SENSING** receptor (CaSR) plays a key role in the regulation of parathyroid hormone (PTH) secretion and hence extracellular Ca homeostasis [1, 2]. The physiological importance of CaSR is confirmed by the identification of CaSR mutations in patients with familial hypocalciuric hypercalcemia (FHH), neonatal severe hyperparathyroidism (NSHPT), autosomal dominant hypocalcemia (ADH) and some cases of sporadic hypoparathyroidism [3–7]. ADH is a disease characterized by hypocalcemia with inappropriately low concentration of PTH and relative

hypercalciuria, which leads to nephrolithiasis and nephrocalcinosis especially after treatment with active vitamin D<sub>3</sub> [5]. Although active vitamin D<sub>3</sub> is indispensable for the treatment of patients with idiopathic hypoparathyroidism (IHP), differential diagnosis between ADH and IHP is clinically sometimes very difficult. The cause of ADH is gain of function mutations in CaSR gene that inhibit PTH secretion and renal Ca reabsorption despite hypoparathyroidism. Twenty-six distinct activating mutations in the CaSR gene have been reported to date in patients with ADH or sporadic hypocalcemia [8]. The functions of mutant CaSR have been analyzed by transfecting mutant cDNAs *in vitro* and measuring intracellular Ca or inositol 1,4,5-trisphosphate in response to various concentrations of extracellular Ca. In the present study, we describe a Japanese

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family with ADH in three generations and show the functional properties of the mutant CaSR found in this family.

### Case report

#### *Patient III-2*

A Japanese male infant, aged 7 days, developed seizures and was referred to Hitachi General Hospital. His mother had had a prior history of breech presentation and caesarian section, and caesarian section was again selected for delivery. He was born at 37 weeks gestation without asphyxia. His body weight was 3265 g at birth and he had no difficulty in drinking mother's milk until then. He had a narrow palate and deformity of left ear lobe, but he did not have thymic hypoplasia or cardiac anomaly. His initial laboratory data revealed marked hypocalcemia (5.2 mg/dl; reference range 8.5–10.2). Serum phosphate (P) was relatively high (8.7 mg/dl) (Table 1). Intact PTH level was 15 pg/ml and inappropriately low for his hypocalcemia (reference range 10–65). He was diagnosed as IHP and treated with up to 0.5 µg/day of 1α-hydroxyvitamin D<sub>3</sub> in combination with 2–3 g/day of calcium lactate. Although urinary electrolytes were not measured before the treatment, he showed hypercalciuria during treatment and bilateral nephrolithiasis developed. He is now under treatment with 0.05 µg/day of alfacalcidol and serum Ca is around 8.0 mg/dl.

#### *Patient III-1*

The three-year-old sister of the proband was discovered to have hypocalcemia during the family survey. Her serum Ca concentration was 7.0 mg/dl, with serum P of 6.3 mg/dl, and an inappropriately low PTH level of 9 pg/ml (Table 1). The ratio of fasting urinary Ca to urinary creatinine (expressed as milligrams of Ca per milligrams of creatinine) (u-Ca/u-Cr ratio) was low (0.01). Her height and weight were normal for her age, and physical examination revealed no abnormality. She was treated with 1 µg/day of alfacalcidol, 25–50 mg/day of hydrochlorothiazide and 3 g/day of calcium carbonate. However, she suffered from recurrent tetany whenever she caught gastroenteritis. She had absolute hypercalciuria during the treatment, although nephrolithiasis and nephrocalcinosis were not detected at 9 years of age. She is now taking 1 µg/day of alfacalcidol and 50 mg/day of hydrochlorothiazide and serum calcium is around 8.5 mg/dl.

#### *Patient II-2*

The 31-yr-old mother of the proband had had a history of tetany, but hypocalcemia was first detected by family screening. Biochemical findings confirmed the presence of hypoparathyroidism as shown in Table 1. Calcifications of the basal ganglia and sub-cortex were detected by computed tomographic scan. Fasting U-Ca/u-Cr ratio was low (0.06) before treatment, but it was in the upper limit of normal range or high during treatment with 1 µg/day of calcitriol and 2 g/day of calcium lactate. Renal ultrasound re-

**Table 1.** Clinical features of the cases at the time of diagnosis

	Reference range*	III-2	III-1	II-2	I-1
Alb (g/dl)	3.4–4.7	4.1	4.0	4.1	3.6
Ca (mg/dl)	8.5–10.2	5.2	7.0	7.0	6.8
P (mg/dl)	2.5–4.5	8.7	6.3	4.8	4.2
Mg (mg/dl)	1.6–2.4	1.7	1.7	1.6	2.0
Cr (mg/dl)	0.5–0.9	0.2	0.2	0.4	1.2
Intact PTH (pg/ml)	10–65	15	9	13	6
Urinary Ca/Cr	<0.2	ND	0.01	0.06	ND

ND: not determined.

Urinary Ca/Cr is expressed as milligrams of Ca per milligrams of creatinine.

\*Reference ranges are determined using healthy adult controls and some values are not applicable to children.

vealed the presence of bilateral nephrolithiasis. She had a history of spontaneous abortion before the first delivery, but two caesarian sections were uneventful.

#### Patient I-1

The grandfather of the proband had not experienced usual signs and symptoms of hypocalcemia. However, when he developed heart failure at the age of 63, he was discovered to have hypoparathyroidism as shown in Table 1. His cardiac function improved after Ca supplementation. He was treated with 0.5 µg/day of calcitriol in combination with 3 g/day of calcium lactate and his serum Ca concentration was kept around 8 mg/dl. Urinary electrolytes were not measured before and during treatment, but bilateral nephrolithiasis was noted by ultrasound examination.

The pedigree of this family is shown in Figure 1. The father of the proband (II-1 in Fig. 1) had normal serum Ca, P and intact PTH concentration. We were unable to obtain blood samples from the uncle (II-3) and grandmother (I-2) of the proband (Fig. 1). However, the uncle had a history of a seizure during infancy.

The hypoparathyroidism exhibited an autosomal dominant inheritance in this family. These findings led us to suspect an abnormality in CaSR gene. Informed consent was obtained from all subjects or their guardians for the studies described below. This

study was approved by the ethics committee of Hitachi General Hospital.

#### Methods

##### DNA extraction and sequencing analysis of CaSR gene

Genomic DNA was extracted from peripheral blood using QIAamp Blood Kit (Qiagen, Hilden, Germany). DNA sequence of CaSR gene was analyzed by PCR amplification of all coding exons of CaSR gene and direct sequencing of PCR products as previously described [9–11]. The PCR products were directly sequenced using PRISM ready reaction dye deoxyterminator cycle sequencing kit and ABI model 373S-36 autosequencer (Perkin Elmer, Chiba, Japan) according to the manufacturer's instructions.

##### Construction of expression vector for wild-type and mutant CaSR

The identified mutation was engineered into wild-type CaSR vector by *in vitro* mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) [9–11]. The primers used were 5'-gacgactatgggctggggattgagaaattc-3' and 5'-gaattcccaatccccagccgccatagtcgtc-3'. The mutated nucleotide was confirmed by DNA sequencing.

##### *In vitro* functional study of the mutant CaSR

Functional property of CaSR was evaluated by measuring inositol 1,4,5-trisphosphate (IP<sub>3</sub>) production after incubating cells expressing the CaSR with various extracellular Ca<sup>2+</sup> concentrations as described [12]. Briefly, COS-1 cells plated in 24-well culture plates were transfected with wild-type or mutant CaSR cDNA. Twenty-four hours later, cells were incubated with 1.5 µCi/well [<sup>3</sup>H]myoinositol (Amersham, Tokyo, Japan) for 16 hours followed by 30 minutes preincubation with PI buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl<sub>2</sub>, 20 mM LiCl in 25 mM PIPES buffer, pH 7.2). Cells were then stimulated with various extracellular Ca concentrations in PI buffer for 30 min. The incubation was terminated by the addition of 1 ml acid-methanol (167 µl HCl in 120 ml methanol). Total

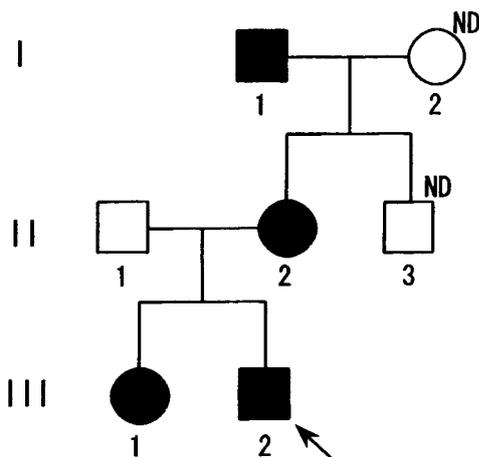


Fig. 1. Pedigree of the family. Arrow denotes the proband and filled symbols indicate affected family members. ND: serum Ca not determined.

[<sup>3</sup>H]inositol phosphate was extracted by ether and applied to ion exchange chromatography using AG1 resin (Bio-Rad, Tokyo, Japan). After inositol monophosphate and inositol bisphosphate were eluted by 0.2 M ammonium formate in 0.1 M formic acid and 0.4 M ammonium formate in 0.1 M formic acid, respectively, IP<sub>3</sub> was eluted by 1.0 mM ammonium formate in 0.1 M formic acid. The radioactivity in IP<sub>3</sub> fraction was counted by liquid scintillation counter. Results were expressed as percentage of maximal IP<sub>3</sub> production that corresponds to IP<sub>3</sub> production of cells with wild-type CaSR exposed to 16 mM extracellular Ca. Statistical significance was evaluated by Student's t-test.

## Results

### Mutation in CaSR gene

Nucleotide sequencing of PCR products from the proband identified a heterozygous CCG to CTG substitution at codon 221 that resulted in the conversion of the wild-type codon for proline to that for leucine (P221L) (Fig. 2). His mother, elder sister, and grandfather had the same heterozygous substitution of this codon (Fig. 2).

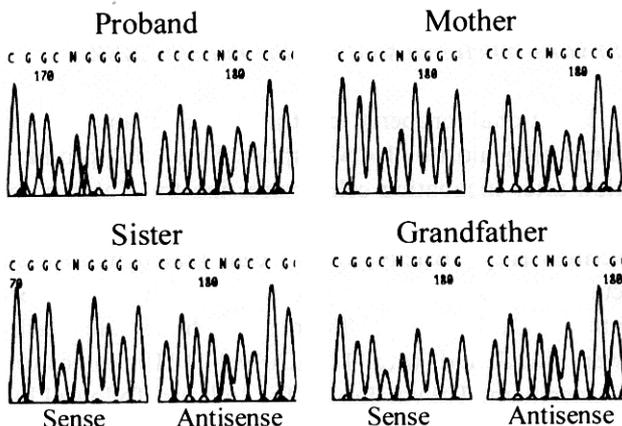


Fig. 2. DNA sequence of CaSR gene in affected family members.

DNA sequence for all coding exons of CaSR gene was determined by directly sequencing PCR products as described in Methods. All affected members showed heterozygous substitution of CTG for CCG at codon 221.

### Functional characterization of P221L mutant CaSR

We studied the function of the mutant P221L CaSR by measuring IP<sub>3</sub> production. As shown in Fig. 3, IP<sub>3</sub> production increased in response to higher extracellular Ca when wild-type CaSR was expressed. When P221L mutant CaSR was expressed, the dose-response curve shifted to the left. IP<sub>3</sub> production in response to 0.5, 1, 2 and 3 mM Ca of cells transfected with P221L mutant CaSR cDNA was significantly higher than that of cells with wild-type CaSR. These results indicate that this P221L mutation is an activating mutation of CaSR.

## Discussion

We have identified an activating mutation of CaSR in a Japanese family. This nucleotide change causes proline to leucine substitution at codon 221, which is in the N-terminal portion of extracellular domain of CaSR. This mutation has been already described in two reports [8, 13]. Since these reports derived from different countries, it is unlikely that this mutation shows a founder effect. Therefore, proline 221 seems to be one of hot spots of mutations in CaSR gene.

Although four patients described here had the same mutation in CaSR gene, clinical presentation of

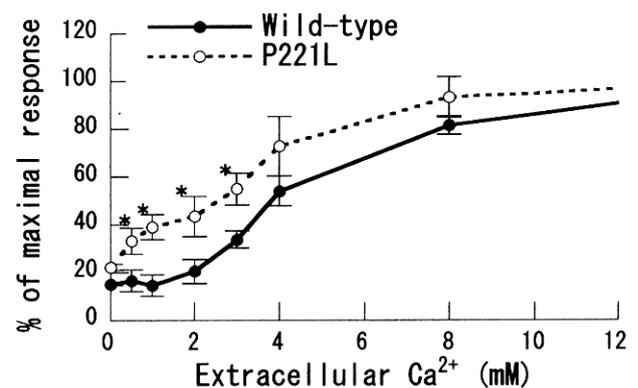


Fig. 3. Functional analysis of the mutant CaSR. Wild-type (●) or the mutant CaSR (○) cDNA was transiently transfected into COS-1 cells and the intracellular IP<sub>3</sub> production in response to various concentrations of extracellular Ca<sup>2+</sup> was evaluated. \*Significantly different from results of wild-type CaSR by Student's t-test.

these patients was quite different. Namely, while the proband showed seizure soon after birth, his grandfather was asymptomatic until adulthood and his mother showed only mild symptoms. Because three patients other than the proband did not need therapy during infancy, it is possible that some coexisting factors contributed to the development of severe hypocalcemia found in the proband. There are several possible causes for hypocalcemia during infancy. For example, administration of magnesium to the mother, small for dates infants and bottle-feeding can be the cause of hypocalcemia of newborns. However, we cannot identify these possible causes of hypocalcemia in this family and it is not clear why he suffered severe hypocalcemia soon after birth. Although it is not written in the medical record, other possible factors could be infection and/or malabsorption due to problems in gastrointestinal tract. Because the amount of active vitamin D<sub>3</sub> necessary for the proband to prevent severe hypocalcemia is not high at present, we speculate that these coexisting factors should have been temporary in nature.

Hypocalcemia causes many symptoms including tetany, seizure, paresthesia and reversible heart failure [14]. Because heart failure in Patient I-1 improved after Ca supplementation and neither valvular nor coronary heart diseases could be detected, it was likely that hypocalcemia at least contributed to the worsening of his heart failure. Although the original patients with ADH were asymptomatic and treatment with active vitamin D<sub>3</sub> was considered to be rather contraindicated considering the high risk of nephrolithiasis and impairment of renal function [3], subsequent study clearly showed that there are patients with ADH who need active vitamin D<sub>3</sub> to prevent symptoms [5]. Therefore, we believe that treatment with active vitamin D<sub>3</sub> should be considered for patients with ADH who have symptoms attributable to hypocalcemia.

Recently, several genes including CaSR, GATA3

and GCMB were shown to be responsible for PTH-deficient hypoparathyroidism [5, 15, 16]. Of these, it was shown that 8 out of 19 unrelated probands with isolated hypoparathyroidism had activating mutations of CaSR suggesting that the frequency of ADH is relatively high [8]. In addition, patients with ADH are sometimes asymptomatic and diagnosed in adulthood as shown in this report. Therefore, it is necessary to differentiate ADH from a disease called IHP. ADH is characterized by relative hypercalciuria that sometimes leads to nephrolithiasis and renal impairment especially after active vitamin D<sub>3</sub> treatment [5]. Actually, although hypercalciuria could not be documented in this family before treatment, bilateral nephrolithiasis was observed in three of four patients after treatment. There is no solid way to distinguish ADH from IHP. Screening of the CaSR gene in all patients with isolated PTH-deficient hypoparathyroidism is impractical. However, it should be considered especially in patients with relative hypercalciuria or with a family history of hypocalcemia. In addition, careful monitoring of urinary Ca excretion during treatment is very important to prevent nephrolithiasis and renal impairment in patients with PTH-deficient hypoparathyroidism.

In conclusion, we have described a family with ADH, and summarized clinical and biochemical data during the treatment retrospectively. Prolonged, careful monitoring of urinary Ca excretion and renal function is necessary in the treatment of PTH-deficient hypoparathyroidism.

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