Novel Gain of Function Mutations of the Calcium-sensing Receptor in Two Patients with PTH-deficient Hypocalcemia

Kishiko Nakajima¹, Kazuko Yamazaki¹, Hironari Kimura¹, Kazue Takano¹, Hitoshi Miyoshi²
and Kanji Sato^{1,3}

Abstract

Among 15 patients with PTH-deficient idiopathic hypocalcemia, we found two novel missense mutations in the calcium-sensing receptor (CaSR). Patient 1, who developed severe hypocalcemia (5.0 mg/dL) and seizures after birth, had a heterozygous *de novo* missense mutation in the transmembrane domain (A844P). The patient is currently receiving a minimum dose of 1 α -OHD₃ (0.5 μ g/day) to maintain the serum calcium level at 6 mg/dL and thus prevent seizures. Patient 2 had asymptomatic hypocalcemia (7.5 mg/dL) and also had a heterozygous missense mutation in the extracellular domain (E228G). These findings suggest that gene analysis of CaSR should be performed in patients with idiopathic hypocalcemia, particularly when it occurs in the neonatal period.

Key words: calcium-sensing receptor, hypoparathyroidism, gain-of-function mutation, hypercalciuria, nephrocalcinosis

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Introduction

The calcium-sensing receptor (CaSR) is highly expressed in the parathyroid gland and kidney, and is directly activated by extracellular Ca, causing inhibition of parathyroid hormone (PTH) secretion and decreased renal Ca reabsorption (1). Since cloning of the CaSR was reported in 1993 (2), more than 270 mutations have been described in the CaSR mutation database (CASRdb; <http://www.casrdb.mcgill.ca>) (3). Most of these mutations are inactivating, loss-of-function mutations, leading usually to benign hypercalcemia (familial hypocalciuric hypercalcemia) but occasionally to neonatal severe primary hyperparathyroidism. Furthermore, nearly 40 gain-of-function mutations have been reported. Patients with this inherited form of hypocalcemia are commonly asymptomatic, but urinary excretion of calcium is greater than that in patients with idiopathic hypoparathyroidism, leading to autosomal dominant hypoparathyroidism (ADH) associated with hypercalciuria (4). However, in some

patients, the hypocalcemia is so severe that it elicits seizures in the neonatal period. Up to the time that ADH was first reported in 1994 (4), numerous cases had been misdiagnosed as idiopathic hypoparathyroidism and may have been treated with an inappropriately high dose of vitamin D, resulting in nephrocalcinosis, nephrolithiasis and chronic renal failure (4-14). During the last 28 years, more than 20 patients with PTH-deficient hypocalcemia without any history of cervical surgery or irradiation have been referred to our Institute. Since the treatment strategy for hypocalcemic patients with idiopathic hypoparathyroidism differs completely from that of patients with gain-of-function CaSR mutation, we performed analysis of the CaSR gene in 15 hypocalcemic patients with PTH deficiency, and found two novel mutations. In one case, the patient suffered severe hypocalcemia from the neonatal period and required careful control with an appropriate dose of vitamin D, whereas in the other, the patient had mild asymptomatic hypocalcemia and required no treatment.

¹Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical University, Tokyo, ²Miyoshi Clinic, Tokyo and ³Field of Pathophysiology and Treatment of Thyroid and Parathyroid Disease, Division of Internal Medicine, Graduate School of Medicine, Tokyo Women's Medical University, Tokyo

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Correspondence to Dr. Kanji Sato, satokan@attglobal.net

Table 1. Clinical Features of 15 Patients with PTH-deficient Hypocalcemia

	Sex	Born	Onset (yrs)	Symptoms	Alb (g/dL)	Ca (mg/dL)	P (mg/dL)	Mg (mEq/L)	Cr (mg/dL)	iPTH (pg/mL)	1,25(OH)2D (pg/mL)	CaSR muta tion	Cf
1	M	1934	59	T	4.7	6.2	5.9	1.8	1.16	<5.0	ND	(-)	
2	M	1936	63	T, CV	4.2	4.2	7.1	1.5	1.11	<5.0	17.6	(-)	
3	M	1939	63	Tr	4.2	6.5	4.4	1.7	0.84	19	22.9	(-)	
4	F	1942	30	T	4.0*	7.6*	3.6*	1.6*	1.01*	<5*	ND	(-)	
5	F	1946	46	T	4.7	6.9	5.1	1.5	1.06	<5.0	ND	(-)	
6	F	1946	53	T, HF	4.0	5.6	6.8	1.5	0.99	<5.0	ND	(-)	
7	M	1947	56	Tr	4.2	6.3	5.2	1.5	0.87	5	23	(-)	
8	F	1948	?	(-)	4.7	7.7	5.1	1.5	0.50	23	44.2	E228G	Case 2
9	F	1959	41	CV,T	4.5	4.2	8.8	1.4	0.97	<5.0	ND	(-)	
10	F	1961	40	(-)	4.1	7.3	4.6	1.5	0.69	19	22.4	(-)	
11	F	1962	11	T	4.3*	6.8*	5.1*	1.5*	0.70*	<5.0*	ND	(-)	APECED
12	F	1967	24	T	4.0*	6.4*	4.5*	1.5*	0.86*	<5.0*	ND	(-)	
13	M	1967	3days	CV,T	4.2*	5.3*	4.4*	1.3*	1.50*	<5.0*	ND	A844P	Case 1
14	F	1969	7	T	4.0*	6.2*	5.1*	1.4*	0.89*	<5.0*	ND	(-)	
15	M	1973	18	T	4.4	5.7	7.0	1.5	0.90	<5.0	ND	(-)	

* Data were obtained when the patients did not take vitamin D for a while.

T: tetany, Tr: Trousseau sign, HF: heart failure, CV: convulsion, ND: not determined.

Case Report

Among 20 patients who were referred to our hospital with idiopathic PTH-deficient hypocalcemia, 15 agreed to investigation of their CaSR (Table 1). All of the patients had hypocalcemia (4.2-7.7 mg/dL), hyperphosphatemia (4.4-8.8 mg/dL), and a low to undetectable serum level of intact PTH (<5 pg/mL to 23 pg/mL). One patient had autoimmune polyendocrinopathy, candidiasis, and ectodermal dermopathy (APECED) (15).

Patient 1: A 35-year-old man patient was referred to our hospital because of repeated seizures due to severe hypocalcemia, nephrocalcinosis and renal dysfunction. The first seizure occurred 3 days after birth, and the serum level of calcium was 5.0 mg/dL. The patient was diagnosed as having idiopathic hypoparathyroidism and had been prescribed 1 α -hydroxyvitamin D₃ (1 α -OHD₃) to maintain the serum calcium level in the normal to low normal range (7-9 mg/dL). He had mental retardation, and had attended special classes from elementary school at 7 years of age.

The seizures were prevented by 1 α -OHD₃ and sodium valproate (depaken, Kyowa-Hakko-Kirin), but the hypercalciuria continued and nephrocalcinosis and calcification of the basal ganglia developed. Although hydrochlorothiazide was administered at a nearby university hospital to decrease the excretion of urinary calcium (12), the patient developed hyperuricemia (12 mg/dL; reference range 3.7-6.9 mg/dL) and was prescribed allopurinol in 1999, when the serum levels of albumin, calcium, phosphate, BUN, and creatinine were 4.6 g/dL (3.8-5.1 g/dL), 8.6 mg/dL (8.5-9.9 mg/dL), 4.8 mg/dL (2.5-4.3 mg/dL), 20.3 mg/dL (8.0-20.0 mg/dL), and 1.8 mg/dL (0.69-1.06 mg/dL), respectively. The urinary concentrations of calcium, uric acid, and creatinine were 10.9 mg/dL, 28 mg/dL, and 48 mg/dL, respectively. Ab-

dominal X-ray examination revealed marked nephrocalcinosis in both kidneys (Fig. 1). When hydrochlorothiazide was discontinued and 1 α -OHD₃ was reduced, the patient developed generalized seizures, and was referred to our hospital in October 2001, accompanied by his mother.

The patient was 165 cm tall and weighed 70 kg. Trousseau sign was evident upon measurement of blood pressure. Neurologically, there were no abnormal findings, but the patient was unable to understand general conversation, although his mother understood her son's intentions. After obtaining informed consent from the mother, gene analysis of CaSR was performed. The serum level of calcium was 9.3 mg/dL in the mother and 9.4 mg/dL in the father, and no mutation of CaSR was found in the parents. After a missense mutation had been identified, the daily dose of 1 α -OHD₃ was reduced from 1-2 μ g to 0.5 μ g in order to maintain the serum calcium level at 6.0-6.4 mg/dL. At this serum calcium concentration, the patient was free of generalized seizures. The serum levels of albumin, calcium, and phosphate were 4.3 g/dL, 6.0-6.4 mg/dL, and 3.7-5.2 mg/dL, respectively, and the serum level of intact PTH was 2 pg/mL (10-65 pg/mL). Total daily urinary excretion of calcium, phosphate, uric acid, BUN and creatinine was 100 mg, 960 mg, 380 mg, 11.3 g, and 1.45 g, respectively, and total urine volume was 5,380 mL/day. The ratio of calcium clearance to creatinine clearance was 0.069, and the serum levels of calcium and creatinine was maintained at this level for 5 years thereafter.

Patient 2: A 54-year-old woman noticed hand tremor, and CT scan of the brain revealed mild calcification of the bilateral basal ganglia. Since the serum level of calcium was decreased, the patient was referred to our institute without any medication in July 2002. Physical examination revealed no abnormality except for the hand tremor, which was diagnosed as essential tremor by a neurologist. There was no

Figure 1. Plain X-ray film of the abdomen in patient 1. Marked nephrocalcinosis is present in both kidneys.

Trousseau sign or Chvostek sign. Serum levels of albumin, calcium, phosphate, and creatinine were 4.7 g/dL, 7.7 mg/dL, 5.1 mg/dL, and 0.50 mg/dL, respectively. Serum levels of intact PTH and 1,25-dihydroxyvitamin D (1,25-(OH)₂D) were 23 pg/mL and 44.2 pg/mL (20-60 pg/mL), respectively. Total daily urinary excretion of calcium, phosphate and creatinine was 230 mg, 790 mg, and 970 mg, respectively (total urine volume 920 mL/day). The ratio of calcium clearance to creatinine clearance was 0.24. A family history could not be obtained from the patient, but she agreed to undergo CaSR gene analysis.

Materials and Methods

DNA amplification and sequence analysis

Informed consent to participate in the study was obtained from the parents of patient 1 and from patient 2. This study was approved by the Ethics Committee of Tokyo Women's Medical University. Samples of venous blood were obtained, and genomic DNA was extracted from leukocytes using a proteinase K-phenol-chloroform procedure as described previously (15). Exons 2-7 of the CaSR gene, encompassing the entire coding sequence, were amplified using polymerase chain reaction (PCR) with previously reported primers (16). After amplification, the PCR products were purified and then sequenced directly with an ABI PRISM Dye Terminator Cycle Sequencing Kit and a 373A automated fluorescence sequencer (PE Applied Biosystems, Foster City, CA), as described previously (15).

Results

Direct sequence analysis of PCR-amplified CaSR exons in patient 1 identified a heterozygous mutation at bp 2,530 (GCC to CCC), with codon 844 changed from alanine to proline (A844P) in transmembrane region 7 encoded by

exon VII (Fig. 2). However, this mutation was not detected in the patient's mother or father, suggesting that the missense mutation had occurred *de novo*. According to the Calcium-Sensing Data Base (<http://www.casrdb.mcdiil.ca/?Topic=CasrMutation>), this is a novel mutation, although a similar mutation (A844T) was reported.

Furthermore, direct sequence analysis of CaSR in patient 2 also revealed a heterozygous mutation at bp 683 (GAG to GGG), with codon 228 changed from glutamic acid to glycine (E228G) in the extracellular domain encoded by exon IV (Fig. 3). Since the patient denied any relevant family history, we were unable to conclude whether this mutation had been inherited or had occurred *de novo*. According to the Calcium-Sensing Data Base (<http://www.casrdb.mcdiil.ca/?Topic=CasrMutation>), this is a novel mutation, although similar mutations (E228Q and E228K) have been reported.

No mutation in Exons 2-7 was found in the other 13 patients, including a patient with APECED.

Discussion

Generally patients with PTH-deficient hypocalcemia of unknown origin are diagnosed as having idiopathic hypoparathyroidism, and treated with active vitamin D. Although urinary excretion of calcium is decreased in idiopathic hypoparathyroidism and relatively increased in patients with gain-of-function mutation of CaSR (1), the differential diagnosis between the two hypocalcemia becomes difficult, particularly after treatment with active vitamin D, since urinary excretion of calcium is increased in both conditions (17). Even if there is no relevant familial history, the differential diagnosis between sporadic hypocalcemia with hypercalciuria due to gain-of-function mutation of the CaSR gene and idiopathic hypoparathyroidism is important because the former patients have constant hypercalciuria and develop renal insufficiency if an inappropriately high dose of vitamin D and calcium supplementation is prescribed (1).

Figure 2. Direct sequencing of the PCR products for exon 7 of the CaSR in patient 1. PCR products for exon 7 of the CaSR from Patient 1 (lower panel) and his father (upper panel) were directly sequenced as described in *Methods*. The left panel shows the sequence of the sense strand, and the right panel shows that of the antisense strand. *N* indicates a heterozygous mutation at codon 844.

This is particularly important for hypocalcemic patients who develop severe hypocalcemia due to PTH deficiency in the neonatal period. When patient 1 was born in 1967, ADH was not known and the patient was diagnosed as having idiopathic hypoparathyroidism. He had been treated with 1α -OHD₃ at a local university hospital so that the serum level of calcium was maintained within the subnormal or slightly hypocalcemic range. However, when serum calcium was maintained at around 8 mg/dL by supplementation of 1α -OHD₃ (1-3 μ g/day), urinary excretion of calcium would have been excessively increased due to activation of CaSR in the nephron, leading to nephrocalcinosis, nephrolithiasis, and chronic renal failure (Fig. 1). Therefore, care should be taken not to overtreat such patients with vitamin D and/or calcium supplements.

The clinical features and gene mutation in patient 1 were very similar to those of an infant patient reported from the University of Hokkaido, Japan (12), who developed intractable seizures 10 days after birth, accompanied by hypocalcemia (1.1 mM), hyperphosphatemia (3.4 mmol/L), and an undetectable serum level of PTH. This patient also had a *de novo* heterozygous mutation (A853E) in the seventh transmembrane domain of the CaSR. Although we could not perform functional studies of the mutant CaSR, a missense mutation in the transmembrane domain of the CaSR would have an important effect on the structure and markedly increase the sensitivity to extracellular Ca²⁺ concentration. To date, nearly 10 cases of sporadic hypercalciuric hypocalcemia due to *de novo* gain-of-function mutations of the CaR gene have been reported (1, 7-14), and the present study confirmed that activating mutations of the CaSR may result in not only asymptomatic hypocalcemia but also severe hypoparathyroidism, manifested as tetany and hypocalcemic

seizures in childhood (7). The patient's mental retardation was the result of hypoxemia during general convulsion that frequently occurred in the neonatal period. The patient had polyuria (3-5 L/day), which have been due to the recommendation of pediatricians to take enough water to prevent nephrolithiasis since childhood. In contrast to this patient, his sister developed normally, and has been doing well with normal urine volume.

At present, patient 1 is taking as low a daily dose of 1α -OHD₃ as possible (0.5 μ g/day) to prevent tetany and generalized convulsion. However, the most recent generalized convulsion occurred when the patient developed acute respiratory infection and fever, and had been advised to take a higher dose of 1α -OHD₃ (up to 1 μ g/day) when acute infectious disease develops again. Since inflammatory cytokines such as interleukin-1 and interleukin-6 stimulate CaSR mRNA expression (18, 19), the hypocalcemia may worsen during acute infection because of multiple factors (20).

In contrast to symptomatic patient 1 with severe hypocalcemia, there is a wide consensus that patients with asymptomatic familial hypocalcemic hypercalciuria should not be treated routinely with vitamin D. After patient 2 had been notified that she had a CaSR point mutation, and told that no treatment was necessary, she has been doing well and has not needed to visit hospital for the last 6 years.

On the basis of a nationwide epidemiologic survey of idiopathic hypoparathyroidism and pseudohypoparathyroidism in Japan, the total numbers of affected patients are estimated to be 900 (690-1,100) and 430 (330-520), respectively (95% confidence intervals in parentheses) (21). However, the diagnosis of idiopathic hypoparathyroidism should be made after excluding a number of related disorders (22).

In summary, we have identified two patients with gain-of-

Figure 3. Direct sequencing of the PCR products for exon 7 of the CaSR in patient 2. PCR products for exon 4 of the CaSR from Patient 2 (lower panel) and a normal subject were directly sequenced as described in *Methods*. The left panel shows the sequence of the sense strand, and the right panel shows that of the antisense strand. N indicates a heterozygous mutation at codon 227.

function mutations of the CaSR among 15 patients with PTH-deficient hypocalcemia. Patient 1 had severely symptomatic hypocalcemia soon after birth and had been treated excessively with active vitamin D₃, resulting in hypercalciuria, renal nephrolithiasis and chronic renal failure associated with mental retardation. The mutation responsible was proved to be a de novo heterozygous mutation in the seventh transmembrane domain of exon 7 (A844P). The patient is now being treated with a minimal dose of 1 α -OHD₃ (0.5 μ g/day) to prevent tetany and generalized convulsion. Patient

2 was proved to have a heterozygous mutation in the extracellular region (E228G); she was asymptomatic and required no treatment. These data suggest that an appreciable number of cases of idiopathic hypoparathyroidism may be due to gain-of-function mutation of the CaSR.

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