Clinical Research Article

Autosomal Dominant Hypocalcemia With Atypical Urine Findings Accompanied by Novel *CaSR* Gene Mutation and VitD Deficiency

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Abbreviations: ADH, autosomal dominant hypocalcemia; Ca, calcium; Ca2+o, extracellular Ca2+ concentration; CaSR, calcium sensing receptor; FECa, fraction excretion of calcium; PCR, polymerase chain reaction; WT, wild-type.

Abstract

Introduction: Autosomal dominant hypocalcemia (ADH) is caused by gain-of-function mutations of the calcium sensing receptor (CaSR). It is characterized by hypercalciuria in spite of hypocalcemia. Vitamin D deficiency increases calcium reabsorption in the distal tubules of the kidneys, resulting in hypocalciuria.

Materials and methods: A 38-year-old female proband had hypocalcemia, hypocalciuria, and vitamin D deficiency. Her father and brother also had hypocalcemia, but her mother was normocalcemic. We analyzed the *CaSR* gene abnormality in this family. Polymerase chain reaction (PCR) and sequence analysis were performed to explore the *CaSR* gene mutation. Mutagenesis, transfection, and functional analysis were performed on the discovered genetic abnormalities.

Result: PCR and sequence analysis revealed that the proband, her father, and brother had a novel heterozygous mutation of the *CaSR* genes that causes threonine to asparagine substitution at codon 186 (T186N). Using HEK293 cells transfected with wild-type or T186N *CaSR* complementary DNA, we assessed the intracellular Ca^{2+} concentration in response to changes in the extracellular Ca^{2+} concentration. The cells transfected mutant *CaSR* gene had higher activity than that of wild-type. Therefore, we determined our patient had ADH with a novel mutation of the *CaSR* gene and hypocalciuria resulting from a vitamin D deficiency. We administered vitamin D to the proband, which caused elevation of her urinary calcium level, a typical finding of ADH.

Conclusion: Vitamin D deficiency was suggested to potentially mask hypercalciuria in ADH. Hypocalcemia with vitamin D deficiency should be diagnosed with care.

The calcium sensing receptor (CaSR) is a G-protein coupled receptor belonging to family C. It is expressed in many cell types, including parathyroid, kidney, and osteoblasts, and has been shown to play a key role in calcium homeostasis. CaSR is activated by an increase in extracellular Ca^{2+} concentration (Ca^{2+} o); it regulates PTH secretion and renal calcium excretion to maintain calcium homeostasis. Autosomal dominant hypocalcemia (ADH) causes hypocalcemia and suppression of PTH secretion because of a *CaSR* activation mutation. Patients with ADH have increased sensitivity to extracellular calcium, impairing the ability to detect and correct hypocalcemia. A typical phenotype of ADH is hypocalcemia with increased urinary excretion of calcium [1].

We report a family with ADH with a novel mutation in *CaSR* gene (T186N). The proband of this pedigree had an atypical phenotype of ADH because of vitamin D deficiency; she had hypocalcemia with normal urine calcium (Ca) excretion. We show her clinical course and the results of functional analysis of the *CaSR* with a novel mutation.

Case Description

The proband, a 38-year-old Japanese woman, had QT prolongation noted during childhood. Hypocalcemia and vitamin D deficiency were identified in a routine blood examination and she was transferred to our hospital for further investigation. Her daily calcium intake was sufficient, and we excluded lack of outdoor activity and excessive UV protection as potential causes. The proband has not had thyroid surgery or cervical radiation therapy, and physical examination detected no obvious abnormalities. Her serum Ca level was 7.3 mg/dL, which was below the normal range (reference range, 8.5-10.2 mg/dL) with normal serum inorganic phosphorus level (4.3 mg/dL, reference range, 2.5-4.5 mg/dL). Her serum 25-hydroxyvitamin D level was deficient at 11.9 ng/mL, but renal function was normal. Although she had hypocalcemia, her plasma PTH level was not elevated (14.7 pg/mL) and urinary Ca excretion was reduced: fraction excretion of calcium (FECa) was 0.86% and urinary Ca/creatinine ratio was 0.12. She had not taken drugs related to calcium metabolism. Physical findings were normal without evidence of rickets or osteomalacia.

The proband's paternal grandmother, father, and brother all had hypocalcemia, and her father and brother were taking vitamin D preparations. The father's serum Ca level was 8.5 mg/dL with normal serum inorganic phosphorus level, his renal function was in the normal range, and his serum PTH level was 9 pg/mL. His FECa was high at 1.63%. Similarly, the proband's younger brother's serum Ca level was 8.3 mg/dL and serum phosphorus was in the normal range, his serum PTH level was 8 pg/mL, and FECa was high at 1.53% (Table 1).

Blood samples from the proband's mother and paternal grandmother were unavailable. We also did not obtain blood samples from other relatives, but they had not been diagnosed with hypocalcemia and had no hypocalcemia-related symptoms, such as tetany or nephrolithiasis. The proband's decreased urinary calcium excretion was inconsistent with

Table 1. Patient clinical characteristics

		Proband (III-2)	Father (II-2)	Brother (III-3)
Vitamin D intake		(-)	(+)	(+)
	Reference range			
Albumin, g/dL	3.4-4.7	4.2	4.3	4.8
Calcium, mg/dL	8.5-10.2	7.3	8.5	8.3
IP, mg/dL	2.5-4.5	4.3	3.6	4.3
Magnesium, mg/dL	1.6-2.4	1.8	1.9	1.8
Creatinine, mg/dL	0.5-0.9	0.54	0.83	0.8
Urinary calcium, mg/dL		12.4	13.4	29
Urinary creatinine, mg/dL		103	80.4	182.7
PTH-intact, pg/mL		14.7	9	8
25(OH)Vit, ng/mL		11.9	NA	NA
Fraction excretion of calcium, %	1-2	0.86	1.63	1.53
Urinary calcium/creatinine	0.05-0.15	0.12	0.17	0.16

Abbreviation: NA, not available.

ADH, but we decided to do a genetic analysis because the family history suggested the possibility of ADH.

Materials and Methods

Informed consent was obtained from the proband and her father, mother, and brother for the collection, analysis, and publication of personal, familial, and clinical data. It was also obtained for performance of genetic tests on leukocytes. This study was performed with approval of Wakayama Medical University Ethics Committee (No.138).

Polymerase Chain Reaction and Sequence Analysis

The genomic DNA was extracted from the peripheral blood leukocytes from the proband and her father, mother, and brother using the QIAamp DNA Blood Midi Kit 51185 (QIAGEN, Hilden, Germany). The genomic DNA were analyzed with polymerase chain reaction (PCR) for all the coding exons of *CaSR*. The primers used were 5′-tcccttgccctggagagacggcaga-3′ and 5′-agagaagagattggcagattaggcc-3′ for exon2; 5′-tacagagcatgccatgaagc-3′ and 5′-cccaaatggtcatgatacagc-3′ for exon3; 5′-ggtgcttaagtgctcactctggagt-3′ and 5′-tagtggcttcatttaaatattgccc-3′ for exon4; 5′-cacgttcctcccactttgtt-3′ and 5′-caaaggccagagagttcagg-3′ for exon5; 5′-atcagaaccaaggacctctg-3′ and 5′-catgcagggccactaaactc-3′ for exon6; and 5′-agtctgtgccacacaataactcactc-3′ and 5′-ggtaggagagctctcggttggtggc-3′, 5′-ttccgcaacacacccattgtcaagg-3′ and 5′-cgatcaggaagcccagggccatgag-3′, 5′-ggaggatgagatcatcttcatcacg-3′ and 5′-ggatcccgtggagcctccaaggctg-3′, 5′-catcgaggaggtgcgttg-3′ and 5′-ccgtggagatctcaatgctt-3′ for exon7. PCR was performed using an AmpliTaq Gold DNA polymerase and Veriti200 Thermal cycler (Thermo Fisher Scientific, Waltham, MA) with the following protocol: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, with a final elongation at 72°C for 10 minutes. The PCR products were sequenced using a BigDye Terminator v3.1/1.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer's instructions.

Mutagenesis, Transfection, and Functional Analysis

To analyze the function of mutant *CaSR*, we created a mutation by in vitro mutagenesis and examined the function of mutant *CaSR* by transient transfection into HEK cells. Wild-type (WT) *CaSR* gene in pcDNA3 expression vector (Invitrogen) was kindly provided by Dr. Seiji F. (Tokushima University, Tokushima, Japan) [2]. In vitro mutagenesis was conducted using KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. To replace the ACC codon encoding Thr (186) with an AAC codon encoding Asn (186), we used 2 primers: 5′-gtctttcctccga*aac*atccccaatgatg-3′ and 5′-catcattgggagt*gt*ttcggaggaaagac-3′. The mutated construct was verified by sequencing.

HEK293 cells were cultured in DMEM (Thermo Fisher Scientific) with 10% FBS. HEK293 cells (1.5×10^5) were divided into 2 groups: WT *CaSR* and mutated *CaSR*, and were transiently transfected using the Lipofectamine LTX Reagent with PLUS Reagent (Thermo Fisher Scientific). The following reagents were mixed as follows: 3.75 μg of pcDNA3 vector (WT or mutated *CaSR*) and 1.5 μL of PLUS reagent were added to 750 μL of Opti-MEM, and the mixture was kept at room temperature for 5 minutes, and then 9.375 μL of LTX reagent was added to the mixture and incubated at room temperature for 20 minutes. These reagents were added to 2 groups of HEK293 cell, respectively, and incubated at 37°C with 5% CO_2 for 48 hours.

The function of the mutated *CaSR* was assessed by measuring intracellular Ca^{2+} concentration using Calcium kit-Fluo 4 (Dojindo Laboratories, Kumamoto, Japan) and Wallac 1420 ARVO MX (PerkinElmer, Billerica, MA). Recording medium was replaced with Ca free medium (20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L MgCl2, 13.8 mmol/L glucose), but everything else was conducted according to the manufacturer's instructions. $CaCl_2$ was added to produce the desired extracellular Ca^{2+} concentration ($Ca^{2+}o$), and the intracellular Ca^{2+} in response to 0, 2.0, 4.0, 8.0, 16, and 32 mmol/L $Ca^{2+}o$ was recorded. We performed 8 independent functional analyses and used the average value of the obtained results. The mean values were corrected by the measured value without the probe (Fluo4). The maximum response of the WT cell was the response to 32 mmol/L $Ca^{2+}o$, so the magnitude of the transient response after an individual stimulus was expressed as a ratio of the response to 32 mmol/L $Ca^{2+}o$.

As an additional study, to analyze the effect of amino acid polarity for *CaSR* function, we transfected p.Thr186 to amino acids other than Asn and examined the function of mutant *CaSR*. Among amino acids that can be expressed by point mutation, we chose Ser (c.557C > G, p.T186S), which is the same neutral amino acid as Thr, and Ile (c.557C > T, p.T186I), which is a hydrophobic amino acid. To replace the ACC codon encoding Thr (186) to an AGC codon encoding Ser (186) and ATC codon encoding Ile (186), we used four primers: 5′-gtctttcctccga*agc*atccccaatgatg-3′ and 5′-catcattgggggat*gct*tcggaggaaagac-3′ for Ser, 5′-gtctttcctccga*atc*atccccaatgatg-3′ and 5′- catcattggggat*gat*tcggaggaaagac-3′ for Ile. In vitro mutagenesis and transfection

were conducted according to the same procedure as that of T186N mutant *CaSR*. In functional study, we added 1.0 mmol/L to the measured $Ca^{2+}o$ to obtain more detailed data; everything else was conducted according to the same procedures.

Statistical Analysis

Data were statistically analyzed using ANOVA test assuming equal variances for functional analysis. $P < 0.05$ was considered statistically significant. Analyses were performed using a commercially available statistical package (JMP Pro version 13.1, SAS Institute, Cary, NC).

Results

Sequence Analysis

Analysis of genomic DNA sequences showed that a heterozygous point mutation of *CaSR* gene was found in Exon4 of *CaSR* gene (c.557C > A, p.Thr186Asn) from the proband, her father, and brother. On the other hand, no mutation of the *CaSR* gene was found in her mother (Fig. 1). As of May 2020, this mutation has not been reported in the public database.

Functional Analysis of T186N, T186S, and T186I Mutant CaSR

The ratio of the response of T186N mutant CaSR cells to $Ca^{2+}o$ 4.0 mmol/L was 1.14 ($\pm$ 0.41), and it was significantly higher than that of WT cells (0.60 $\pm$ 0.42) ($P < 0.005$). The ratio of the response to other $Ca^{2+}o$ concentration showed no statistical difference, but T186N mutant cells tended to be more sensitive than WT cells (Fig. 2).

Similar to the result of functional analysis of T186N mutant *CaSR* cells, T186S and T186I mutant cells tended to have higher ratio of response to $Ca^{2+}o$ 32 mmol/L than that of WT cells. In particular, the response ratio at $Ca^{2+}o$ 4.0 mmol/L was 3.27 $\pm$ 1.13 (T186I) and 2.62 $\pm$ 0.89 (T186S), which were both significantly higher than that of WT cells (0.14 $\pm$ 0.08, $P < 0.01$, Fig. 3).

Clinical Course

We confirmed by functional analysis that the T186N mutation was a gain-of-function mutation and was the causative gene of ADH. Oral administration of vitamin D (calcitriol, 0.5 µg/d) was begun for the proband, FECa level then increased from 0.86% (day 0) to 1.65% (day 238), and PTH-intact level decreased from 14.7 pg/mL (day 0) to 8.5 pg/mL (day 238). Serum Ca concentrations remained largely unchanged. The typical findings of ADH were obtained. (Fig. 4)

Discussion

In this case, urinary Ca excretion did not increase, despite the p.T186N mutant *CaSR* gene being a gain-of-function mutation. Supplementation with vitamin D increased urinary calcium excretion, resulting in hypercalciuria, a typical finding of ADH. Vitamin D deficiency enhances PTH secretion and increases Ca reabsorption in the distal
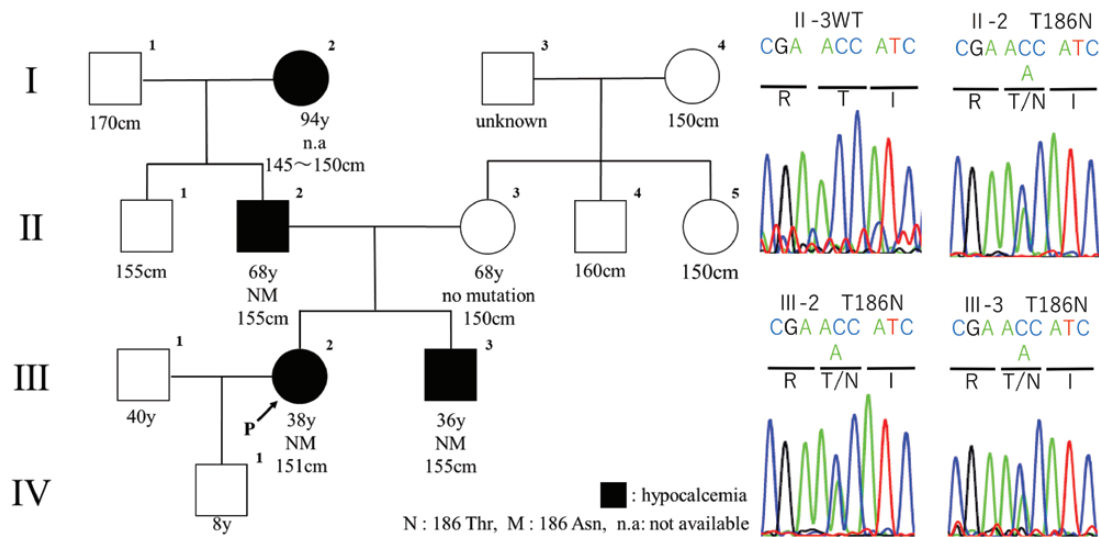

Figure 1. Family tree of the proband (III-2) and direct sequencing of PCR products for exon4 of CaSR. Left panel shows the family tree of the proband. Squares, male; circles, female; white, normocalcemia or no data; black, hypocalcemia. Right panel shows the results of the sequence analysis. PCR products for exon 4 of CaSR from the proband (III-2), her father (II-2), mother (II-3), and brother (III-3) were directly sequenced, as described in the Materials and Methods section. The proband, her father, and brother had heterozygous mutation at codon 186 changing threonine to asparagine, and her mother had wild-type CaSR. Abbreviations: CaSR, calcium sensing receptor; PCR, polymerase chain reaction.

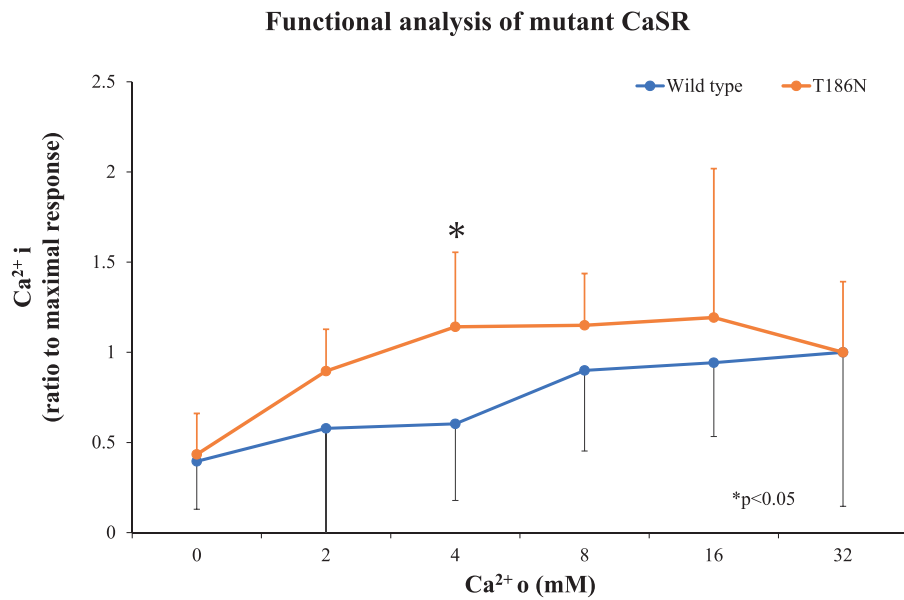## Functional analysis of mutant CaSR

Figure 2. Functional analysis of T186N mutant CaSR. T186N mutant CaSR cDNA was created by in vitro mutagenesis. Wild-type and mutant CaSR cDNAs were transiently transfected into HEK293 cells, and intracellular Ca^{2+} concentration was assessed in response to changes in the extracellular Ca^{2+} concentration as described in the Materials and Methods section. Blue line: wild-type with negative SD, orange line: T186N mutation with positive SD; $*P < 0.05$. Abbreviation: CaSR, calcium sensing receptor.

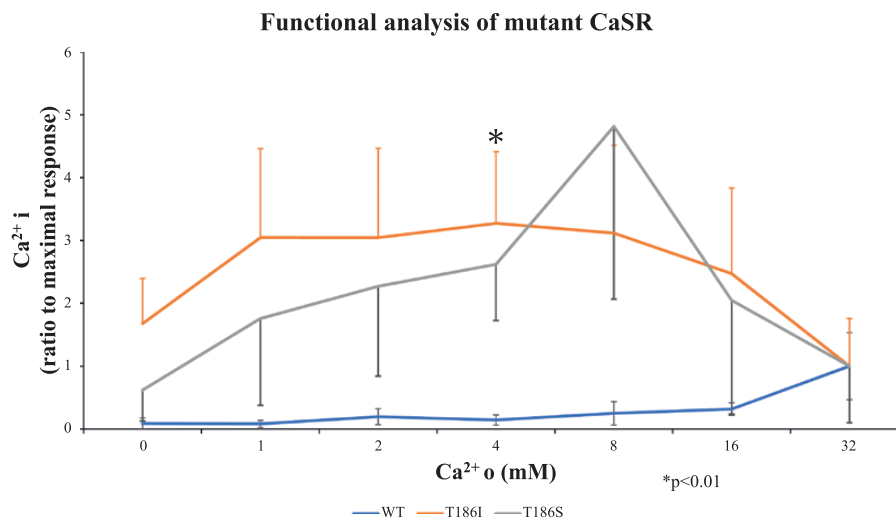## Functional analysis of mutant CaSR

Figure 3. Functional analysis of T186I and T186S mutant CaSR. To analyze the effect of amino acid polarity, T186I and T186S mutant CaSR cDNAs were transiently transfected into HEK293 cells, and a functional study was performed as described in the Materials and Methods section. Blue line, wild-type with positive and negative SD; orange line, T186I mutation with positive SD; gray line, T186S mutation with negative SD, $*P < 0.01$. CaSR, calcium sensing receptor.

tubules of the kidneys [3]. Vitamin D deficiency could mask hypercalciuria in ADH, and some patients with ADH have been reported to have Bartter syndrome, but there were no findings suggestive of Bartter syndrome in this case, such as hypokalemia or metabolic alkalosis.

Vitamin D deficiency is a common disease. There are reports that 20% to 100% of older men and women in the United States, Canada, and Europe, and 32% of healthy students and doctors in Boston hospitals have vitamin D deficiency [3]. In Japan, Miyamoto et al. reported that among women aged 39 to 49 years, 31.6% had 25-hydroxyvitamin D deficiency (<20 ng/mL), 47.4% had suboptimal level (20-30 ng/mL), and only 21% had normal level (>30 ng/mL) [4]. Causes of vitamin D deficiency include length and timing of sun exposure, the amount of skin exposed to sunlight, application of sunscreen, insufficient intake of vitamin D, and abnormalities associated with chronic kidney disease [5]. In this case, the proband did not have excessive sunlight avoidance or an unbalanced diet, and her renal function was normal. Her serum
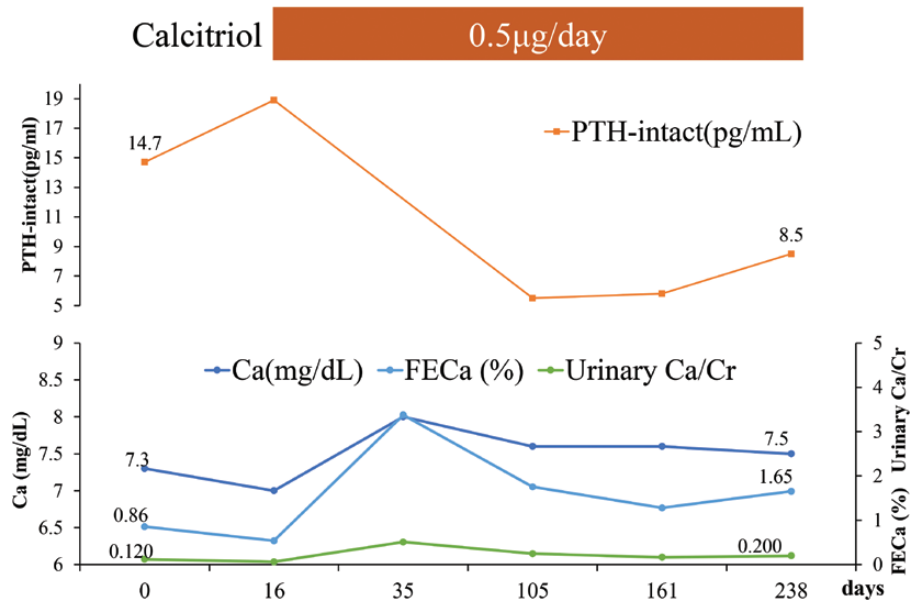
Figure 4. Proband's clinical course. Upper panel shows serum PTH level and lower panel shows serum Ca level, FECa, and urinary Ca/Cre ratio. After supplementation of calcitriol, serum PTH level decreased and FECa increased, a typical finding of ADH. Serum Ca level remained largely unchanged. ADH, autosomal dominant hypocalcemia; FECa, fraction excretion of calcium.

25-hydroxyvitamin D level, however, was low. Insufficient sunlight hours or vitamin D intake may have occurred unconsciously.

There are several reports on the relationship between vitamin D and urinary calcium excretion. Kim et al. reported that serum 1,25 (OH) 2D3 levels correlated positively with urinary calcium excretion in a study of 326 patients with a history of urolithiasis and 163 controls [6]. Hess and Jaeger (1993) also reported that serum 1,25 (OH) 2D3 levels in patients with idiopathic hypercalciuria were higher than in patients with renal stones and normocalciuria [7]. On the other hand, there are reports that supplementing vitamin D for patients with vitamin D deficiency did not affect urinary calcium excretion [8, 9].

There is no consensus on the relationship between vitamin D and urinary calcium excretion, but these reports represent a group with normocalcemia and CaSR was not tested, so patients with mutant *CaSR* gene might have a different course.

To our knowledge, there are no previous reports of an association between ADH and vitamin D deficiency. In this case, diagnosis of ADH before vitamin D supplementation was difficult because of hypocalciuria. Vitamin D deficiency is a common disease, so there may be a number of people with both ADH and vitamin D deficiency that have not been diagnosed. The lack of reports suggests that diagnosis of ADH may have been missed if the patient has both ADH and vitamin D deficiency. In patients with hypocalcemia with vitamin D deficiency, urine and biochemical tests should be performed

again after vitamin D supplementation so diagnosis can be reconsidered.

The p.T186N mutation in CaSR gene is a novel gain-of-function mutation. HEK cells transfected with the p.T186N mutant CaSR gene had higher activity than WT cells. More than 80 residues of *CaSR* mutations have been reported [10, 11]. Among them, gain-of-function mutations of more than 40 residues were reported, and loss-of-function mutations of more than 40 residues were also reported. Adjacent mutations have been reported to have different functions, such as being inactive at residue 227, being active at residue 228, being inactive at residue 680, and being active at residue 681. The mutation we found at residue 186 was an active mutation, but the mutation at residue 185 was inactive. To examine the effect of amino acid polarity on function at residue 186, we made a p.T186S mutation, which is the same neutral amino acid as Thr, and p.T186I mutation, a hydrophobic amino acid, and performed functional analysis. Both mutations were active, so it was suggested that the function of *CaSR* was not affected by amino acid polarity at residue 186. Similarly, at residue 228, 3 active mutations (p.E228G/K/Q) have been reported, and the 4 amino acids have different polarities (E, acidic; G, hydrophobic; K, basic; Q, neutral). On the other hand, P221L is an active mutation (P · L: hydrophobic) and P221Q/S are inactive mutations (Q · S: neutral). Furthermore, L173F is an active mutation and L173P is an inactive mutation, and L, F, and P are all hydrophobic amino acids. It is therefore suggested that the function of *CaSR* is not determined by the mutation site and the

polarity of amino acids, but by the change in the 3-dimensional structure.

The structure of CaSR has been reported in numerous studies. CaSR is a G protein–coupled receptor and functions as a disulfide-tethered homodimer composed of 3 main domains, the Venus flytrap module, cysteine-rich domain, and the 7-helix transmembrane region [12, 13]. The principal agonist of CaSR is known to be extracellular Ca^{2+} [14]. Ligand binding to CaSR occurs within a Venus flytrap module that consists of 2 domains (LB1 and LB2) [15]. Aromatic and aliphatic L-amino acids, such as L-Phe and L-Trp, are positive allosteric modulators of the receptor that increase the sensitivity of CaSR to Ca^{2+} [16]. Previous studies have predicted 5 calcium-binding sites. Calcium-binding site 1 is composed of residues L51, T145, S170, Y218, and S272, and is located in the hinge region between LB1 and LB2 [17]. The putative amino acid binding site is formed by residues Lys47, Leu51, Trp70, Thr145, Gly146, Ser169, Ser170, Ile187, Tyr218, Ser272, His413, and Arg415 [18]. It is also located in the hinge region and partially overlaps with the residues that compose calcium-binding site 1. In addition, several residues (eg, R172, L173, D215, R220) are located in the dimer interface [19], and the L173 and P221 residues have been examined in detail in this region. Zhang et al. reported that the L173P and P221Q mutants produce a receptor with reduced positive homotropic cooperativity and impaired capability to sense Ca^{2+}o, and the L173F and P221L mutants enhance the sensitivity of the CaSR to Ca^{2+}o [20].

We speculate that the residue T186 is located in the hinge region near the predicted calcium-binding site 1 and the putative amino acid binding site. Residue T186 is close to the binding site of Ca^{2+} and L-Phe, which may have affected these structures. In addition, because T186N, T186S, and T186I were gain-of-function mutations, it was suggested that the T186 residue is important for restraining the CaSR from assuming its active form.

As a limitation of this study, it was initially impossible to rule out factors that affect the clinical course other than vitamin D supplementation because of the absence of records of the proband's diet and sun exposure. Second, we have no data about the proband's father and brother from before vitamin D supplementation, so this study is limited to 1 case report and does not allow a generalization of the association between ADH and vitamin D deficiency. Finally, hereditary diseases that can cause hypocalcemia other than CaSR abnormalities include ADH type 2 (GNA11), familial solitary hypocalcemia (GCM2), DiGeorge syndrome (TBX1), HDR syndrome (GATA3), Kenny-Caffey syndrome (TBCE, FAM111A), Kearns-Sayre syndrome (POLG), and others, but the proband's family history and clinical course did not match these diseases. With the exception of CaSR, we have not investigated these genetic abnormalities.

Conclusion

We have described a novel gain-of-function mutation in *CaSR* gene. Vitamin D deficiency decreases urinary Ca excretion and could mask hypercalciuria in ADH. Vitamin D deficiency is a common disease, so it is presumed that there are many cases in which hypercalciuria does not occur, even with active mutation of *CaSR*. Laboratory tests should be performed again after vitamin D supplementation so the diagnosis can be reconsidered. We hope to study similar cases to elucidate the relationship between ADH and vitamin D deficiency.

Additional Information

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Disclosures: The authors have nothing to disclose.

Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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