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Identification and functional analysis of novel calcium-sensing receptor gene mutation in familial hypocalciuric hypercalcemia

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Abstract. Familial hypocalciuric hypercalcemia (FHH) is a benign disorder with heterozygous inactivating mutations in the calcium-sensing receptor (CASR) gene. The present study describes the identification and functional analysis of a novel CASR gene mutation leading to FHH. The proband is a 33-yr-old woman (Ca 11.0 mg/dL, intact-PTH 68 pg/mL, FECa 0.17 %). Leukocyte DNA was isolated in four family members and a novel heterozygous mutation (D190G, GAT>GGT) in exon 4 of CASR gene was identified by direct sequence analysis. The mutant CASR expression vector was constructed by mutagenesis procedure and its response to Ca²⁺ was characterized by transient transfection into human embryonic kidney (HEK) 293 cells and treatment with increasing extracellular Ca²⁺ concentrations. HEK cells didn't activate intracellular signaling (MAPK activation) in response to increases of extracellular Ca²⁺ concentrations when the mutant receptor was expressed normally at the cell surface. The novel heterozygous mutation (D190G) identified in the present study showed that the reduction of activity of CASR to extracellular Ca²⁺ caused FHH in patients and our study demonstrated the importance of Asp-190 participated in response to Ca²⁺ in CASR.

Key words: Calcium-sensing receptor, Familial hypocalciuric hypercalcemia, MAPK signaling pathway

CALCIUM-SENSING receptor (CASR) is the seven-transmembrane G-protein-coupled receptor and CASR gene has six exons (exons 2-7) encoding the CASR protein of 1078 amino acids [1]. CASR is expressed mainly in chief cells of parathyroid glands, C-cells of thyroid and cells lining the kidney tubule. It senses small changes in circulating calcium level and modulates PTH and calcitonin secretion as well as renal Ca²⁺ reabsorption, leading to restoring circulating Ca²⁺ levels to normal [2].

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant-trait benign disorder with heterozygous inactivating mutations in CASR gene. It is characterized by normal or slightly elevated serum intact PTH levels although hypercalcemia and marked decrease in calcium clearance in kidney [3, 4]. In the

present report, we describe identification and functional analysis of a novel mutation leading to FHH.

Patients and Methods

Patients

This study included four family members (I-1, II-1, III-1, and III-2 in Fig. 1) from Hokkaido University Hospital and Sapporo Kosei Hospital. The biochemical characteristics of them are described in Table 1. The proband, individual II-1, was a 33-yr-old woman and her serum adjusted calcium concentrations were elevated (11.0 mg/dL) whereas serum inorganic phosphate was normal (2.8 mg/dL) and serum intact PTH levels were elevated frankly (68 pg/mL). Although enlargement of one parathyroid gland was suspected with

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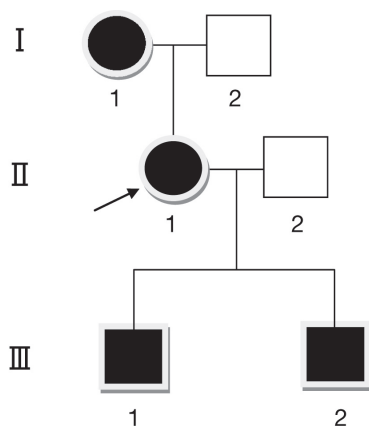
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Abbreviation: FHH; familial hypocalciuric hypercalcemia, CASR; calcium-sensing receptor, HEK; human embryonic kidney, MAPK; mitogen-activated protein kinase, PTH; parathyroid hormone.

Table 1 Biochemical characteristics of the family members

Pedigree	Serum adjusted calcium (8.7-10.3mg/dL) ^a	Serum inorganic phosphate (2.5-4.7mg/dL)	Serum intact PTH (10-65pg/mL)	U _{Ca} /U _{Cr} ratio (mg/mg · cr) ^b	FECa (%) ^c
I-1	11.0	2.4	47	ND	ND
II-1	11.0	2.8	68	0.032	0.17
III-1	10.9	3.7	ND	0.150	0.40
III-2	11.3	3.7	ND	0.186	0.46

ND, Not determined. ^a Normal ranges are given in parentheses. ^b U_{Ca}/U_{Cr} and ^c (U_{Ca}/P_{Ca})/(U_{Cr}/P_{Cr}) where U_{Ca}, U_{Cr}, P_{Ca}, P_{Cr} are the casual urinary concentrations of calcium and creatinine and the serum adjusted calcium and creatinine concentrations, respectively.

**Fig. 1** Pedigree of the family with FHH. Clinical status is indicated by open symbols (unaffected) and solid symbols (affected). The proband is indicated by the arrow.

ultrasonography, ^{99m}Tc-MIBI scan was clearly negative for the gland. Though the 60-yr-old mother's (I-1, Fig.1) serum PTH concentration was not suppressed (47 pg/mL), she was hypercalcemic (11.0 mg/dL). Serum adjusted calcium concentration of her sons (III-1, III-2) was also hypercalcemic (10.9, and 11.3 mg/dL, respectively). Genetic analysis was performed following the approval of the institutional review board and written informed consent by the patients.

PCR

Leukocyte DNA was isolated using the FlexiGene DNA Kit according to the manufacturer's protocol (QIAGEN, Tokyo, Japan). Protein-coding exons 2-7 of the CASR gene were amplified by PCR with nine primer pairs as described in Table 2. PCR conditions were as follows: preheat denaturing at 94°C for 9 min, followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72

Table 2 Primer sequences for sequencing of CASR gene

Exon	Forward and reverse primer (5'-3')
2	ATCCCTTGCCCTGGAGAGACGGC AGAGAAGAGATTGGCAGATTAGGCC
3	AGCTTCCCATTCTTCTCCACTTCTT CCCGTCTGAGAAGGCTTGAGTACCT
4a	ACTCATTACCATGTTCTTGGTTCT GCTGTTGCTAAACCTGTCGC
4b	CCCAGGAAGTCTGTCCACAATG CCCAACTCTGCTTTATTATACAGCA
5	GGCTTGTAATCTTCTTGTCTCCTC GACATCTGGTTTTCTGATGGACAGC
6	CAAGGACCTCTGGACCTCCCTTTGC GACCAAGCCCTGCACAGTGCCCAAG
7a	AGTCTGTGCCACACAATAACTCACTC CTTGTTGAAGAAGATGCACGCCA
7b	TGCTCATCTTCTTCATCGTCTGG CTCTCTGCATTCTCCCTAGCCCAGT

°C for 90 sec for exon 2~5, and preheat denaturing at 94°C for 9 min, followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 90 sec for exon 6~7b. Then, PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan).

Direct sequence analysis

After PCR, purified products were directly sequenced using BigDye XTerminatorTM Purification Kit with ABI PRISM 3100/3100-Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Construction of c-myc-tagged CASR vector

CASR expression (wild-type) vector was generated by inserting a PCR-amplified CASR cDNA fragment into pcDNA3.1/V5-His-TOPO (Invitrogen, Tokyo, Japan). Then, a modified CASR cDNA was constructed with a c-myc epitope tag inserted between ami-

no acids 22 and 23 using the KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) just after the signal peptide cleavage site at amino acid 19 in the CASR NH₂-terminal region. For the insertion, primers used were; forward primer was 5'-GAACAAAACT-GATTTCAGAAGAGGATCTGGTTCGACGAC-CAGCGAGCCCCAAAAGAAGGGG-3' in which the bolded sequence was a c-myc epitope, the underlined sequence was a *Sal I* restriction enzyme site followed by +67 to +90 of the CASR cDNA (where +1 represents the A of the ATG initiation codon). Reverse primer was 5'-TGGCCCGTAGGCAGAGGTGTG-3' followed by +66 to +46 of the CASR cDNA. After the digestion of template plasmid with *Dpn I*, PCR products were self-ligated by T4 polynucleotide kinase and ligase. An insertion of the c-myc sequence into the correct point was confirmed by sequencing.

Site-directed mutagenesis

A mutation of c-myc-tagged CASR vector was generated using the KOD -Plus- Mutagenesis Kit according to the manufacturer's protocol. Primers used were; forward primer was 5'-AATGGTGAGCACCA-GGCCACTGCCATGGCA-3' in which the underlined sequence showed mutated sites. Reverse primer was 5'-GGGGATGGTTCGGAGGAAAGACTTGAAT-TG-3'. The correctness of constructs was confirmed by sequencing.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells, purchased from ATCC, were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 5% Penicillin/Streptomycin. Plasmid vectors (empty, wild-type CASR, c-myc-tagged CASR and D190G vectors) were transfected into HEK293 cells at 60-80% confluent using FuGENE6 Transfection Reagent (Roche, Tokyo, Japan) according to manufacturer's protocol. Forty-eight hours after transfection, cell proteins were extracted.

Extraction of cell proteins and western blot analysis

PBS-washed cells were harvested and lysed in lysis buffer (25 mM HEPES-Tris(pH 7.4)/20% glycerol with protease inhibitor). Cells were homogenized using 1mL syringe and centrifuged for 5 min at 1000 rpm, 4°C to separate nuclei on pellet from cell lysates. Then, the supernatant was ultracentrifuged for 30

min at 15000 rpm, 4°C to separate membrane proteins from cytosol proteins. Membrane protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% gel), and electrotransferred to a PVDF membrane (Millipore, Tokyo, Japan). The membrane was incubated with c-Myc Epitope Tag (9E10) (Thermo, Kanagawa, Japan) and detected by ECL Advance Western Blotting Detection Kit (GE Healthcare, Tokyo, Japan).

The ability of wild-type and mutant CASR to activate the MAPK signaling cascade was assessed by examining the phosphorylation of ERK1/2 with western blot analysis. After transfection, cells were treated with DMEM (increasing CaCl₂ concentration: 0.5-10-mM) for 10min. Whole protein extracts were separated by SDS-PAGE (10% gel) and electrotransferred to a PVDF membrane. The membrane was incubated with Phospho-p44/42 MAP Kinase (Thr202/Tyr204) Antibody (Cell Signaling Technology, Boston, USA) for phosphorylated ERK1/2 or p44/42 MAP Kinase Antibody (Cell Signaling Technology) for total ERK1/2.

Results

Identification of a novel CASR mutation

A heterozygous mutation (D190G, GAT>GGT) was identified in exon 4 of CASR gene encoding CASR extracellular domain by direct sequence analysis (Fig. 2). The proband (II-1), her mother (I-1) and two sons (III-1, 2) were ascertained to have the same mutation.

Cell membrane expression of c-myc-tagged and mutant CASRs in HEK293

c-myc-tagged and mutant CASR expression vector carrying D190G mutation were transfected into HEK293 cells and membrane proteins were extracted. The D190G mutant CASR exhibited the same pattern as c-myc-tagged CASR (Fig. 3), indicating that the D190G mutant CASR is normally expressed in cell membrane. The immature glycosylated CASR is indicated as 140 kDa species, the mature glycosylated as 160 kDa species and the dimerized CASR as over 280 kDa species.

The response of mutant CASR to extracellular Ca²⁺ level

The phosphorylation of ERK1/2 was estimated both in c-myc-tagged and D190G-carrying CASR-expressing HEK293 cells according to stepwise

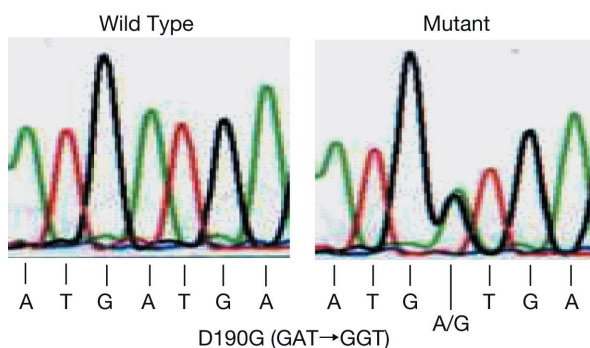


Fig. 2 Identification of the mutation in the CASR gene. Direct sequence analysis revealed a heterozygous missense mutation in the exon 4. The sequence of the sense strand is shown.

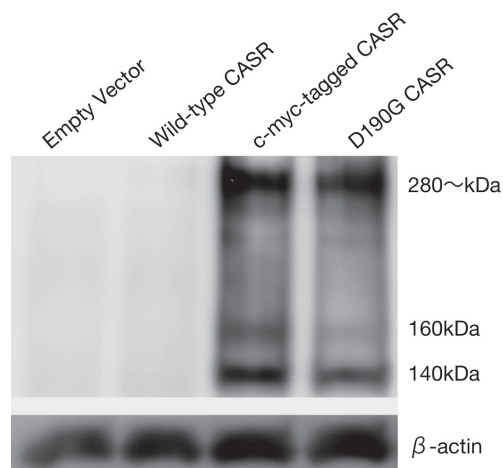


Fig. 3 Western blot analysis of cell membrane proteins extracted from HEK293 cells. HEK293 cells were transiently transfected with empty, Wild-type CASR, c-myc-tagged CASR and mutant (D190G) CASR vectors. Recombinant proteins were stained with c-Myc Epitope Tag (9E10) and β -actin antibody as a loading control.

increase of the extracellular Ca^{2+} concentrations from 0.5 to 10mM. HEK293 cells transfected with D190G vector failed to induce the phosphorylation of ERK1/2 (Fig. 4), indicating declined response to extracellular Ca^{2+} concentration.

Discussion

It is well known that mutations of CASR gene give rise to a variety of disorders by the disturbance of Ca^{2+} homeostasis. Although heterozygous loss-of-function mutations lead to FHH, homozygous mutations cause neonatal severe hyperparathyroidism (NSHPT) which is characterized by marked hypercalcemia, skeletal

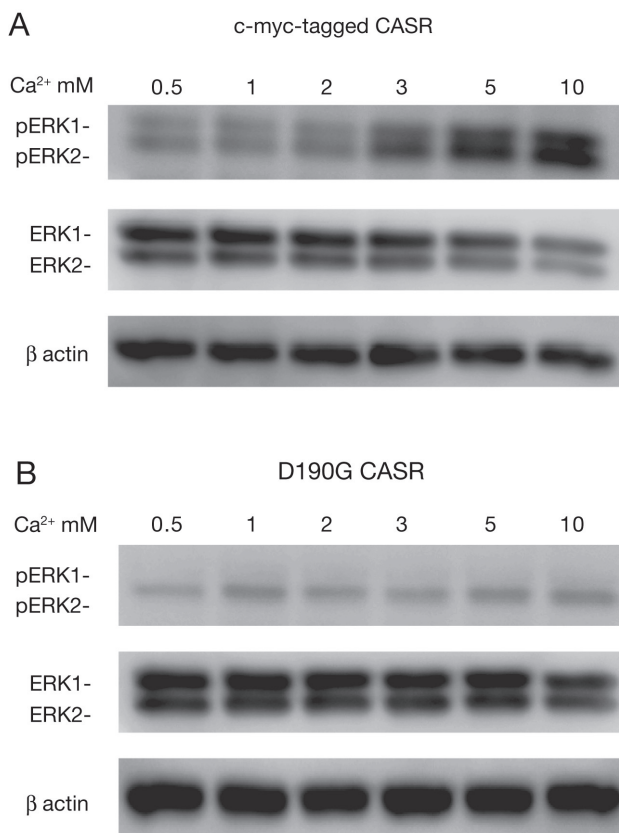


Fig. 4 Phosphorylation of endogenous ERK1/2 activated extracellular Ca^{2+} in HEK293 cells. HEK293 cells were transiently transfected with c-myc-tagged CASR vector (A) and mutant (D190G) vector (B). Forty-eight hours after transfection, the cells were exposed to 0.5-10mM Ca^{2+} for 10min, whole protein extracts were immunoblotted. Western blots were stained with antibodies against either phospho- or total ERK1/2 (marked as pERK or ERK respectively) and β -actin as a loading control.

changes and parathyroid hyperplasia, and requires parathyroidectomy [5, 6]. Heterozygous activating mutations give rise to autosomal dominant hypocalcemia (ADH) which shows hypocalcemia with inappropriate low PTH levels and may be asymptomatic or present with seizures in the neonatal period or childhood [7, 8]. Until now, over 200 unique mutations in the CASR gene have been reported, and over 130 inactivating mutations in FHH/NSHPT and over 60 activating mutations in ADH have been identified [2]. Whereas mutations are scattered throughout all exons of CASR gene, inactivating mutations tend to cluster in particular regions of the extracellular domain (ECD) (amino acids 158-227, 549-607) and the transmem-

brane domain (TMD). Activating mutations also have a tendency to cluster in the ECD (amino acids 116-131) and the TMD (amino acids 788-845) [2].

The ECD of CASR is comprised of amino acid sequences homologous to bacterial periplasmic amino acid-binding proteins which are known as the crystallographic structures [9] and have a Venus-Flytrap (VFT) structure composed of two lobes bound by a hinge region of three interwoven strands [2]. This has been confirmed by 3D analysis of the metabotropic glutamate receptor 1 (mGluR1) [10] and homologous model of the CASR ECD (amino acids 23-528) was deduced [11-13]. Almost all amino acids composing mGluR1 VFT and participating in glutamate binding are identified in the CASR ECD [14]. It was suggested that Ca^{2+} binding site (site1) in the CASR consists of polar residues; Ser-170, Asp-190, Gln-193, Ser-296 and Glu-297 directly concerned in Ca^{2+} coordination, and an additional set of residues; Phe-270, Tyr-218 and Ser-147 involved in the coordination sphere of the cation [15]. Furthermore, another study indicated that five potential Ca^{2+} binding sites (site1-5) were present in the CASR VFT [16].

The CASR is a G protein-coupled receptor inducing intracellular signals via multiple G proteins. Stimulated CASR couples to G_q proteins producing phospholipase C (PLC)-mediated diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) with mobilization of intracellular Ca^{2+} and activation of protein kinase C (PKC). Stimulated CASR also couples to G_i proteins which suppress cyclic AMP (cAMP). The mitogen-activated protein kinase (MAPK) is the serine/threonine kinases with molecular weight of about 40,000 including extracellular signal-regulated kinase

(ERK1/2), p38 MAP kinase (p38) and c-Jun N-kinase (JNK). The signals via both G_q and G_i proteins by the activated CASR have been implicated in ERK1/ERK2 phosphorylation. The stimulated CASR can also activate p38 and JNK [17].

In the present study, we identified the novel mutation, Asp190Gly, located in Ca^{2+} binding site (site1) of the CASR gene. We clarified that the CASR carrying this mutation decreased the activity of MAPK phosphorylation, whereas its expression level at the cell membrane was normal (Fig 3). Zhang *et al.* [18] proposed that the responsiveness of CASR with Asp-190 mutation to Ca^{2+} decreased in alanine assay. Glycine as well as alanine is a neutral amino acid in the classification scheme, and their chemical characteristics are generally similar. These facts suggest that the attenuation of CASR VFT binding strength to Ca^{2+} due to the substitution from Asp-190 to Gly-190 causes FHH. Although it was advocated that the artificial substitution of Asp-190 led to the abnormalities of CASR function *in vitro*, the present case, for the first time, showed functional role of this site *in vivo*. Our study demonstrated the significance of Asp-190 involved in response to Ca^{2+} in CASR VFT site1.

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