The Effect of Calcium-Sensing Receptor Gene Polymorphisms on Serum Calcium Levels: A Familial Hypocalciuric Hypercalcemia Family without Mutation in the Calcium-Sensing Receptor Gene

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Abstract. Familial hypocalciuric hypercalcemia (FHH) is a benign syndrome with elevated levels of serum calcium, relative hypocalciuria, and non-suppressed serum parathyroid hormone (PTH) levels. FHH usually occurs by a heterozygous mutation of the calcium sensing receptor (Casr), but some FHH patients show no mutations of the Casr. We encountered a unique FHH family in which the proband and her mother had many calcium deposits on their skin. The proband was medicated with Levothyroxine for hypothyroidism due to an iodine transport defect (ITD). We searched for mutation of the Casr, but found none. The only change distinguishing the proband and her mother from her father was at codon 990, reported to be a polymorphic site. We investigated the frequency of polymorphism at codon 990, but a significant relationship between the three genotypes and the serum calcium concentration was excluded. At the other polymorphic sites at codon 536, 926, 986, and 1011, polymorphisms were rare in Japanese, and we could not confirm a significant relationship. In conclusion, mutation in the Casr gene alone does not explain all cases of FHH. The other mechanisms should be identified.

Key words: Familial hypocalciuric hypercalcemia (FHH), Calcium-sensing receptor (Casr), Polymorphism

Familial hypocalciuric hypercalcemia (FHH) is a benign clinical syndrome with elevated levels of serum calcium and to a lesser extent of magnesium, relative hypocalciuria, and non-suppressed serum parathyroid hormone (PTH) levels. In previous linkage studies of FHH, some genes were reported to be candidates for the gene responsible for FHH, and in most cases examined, the mutations were mapped to chromosome 3q13.3-q21. After the human calcium-sensing receptor (Csr) was cloned, mutations of the Csr were reported in cases of FHH and in neonatal severe hyperparathyroidism (NSHPT). Csr+/- mice, in which the Csr is absent in heterozygotes, show features analogous to those of FHH [1], i.e., benign and modest elevations of serum calcium, magnesium and PTH as well as hypocalciuria. In contrast, Csr-/- mice, like humans with NSHPT, have markedly elevated levels of serum calcium and PTH, parathyroid hyperplasia, bone abnormalities, retarded growth and premature death. These findings indicated that modest-severe elevations of the serum calcium concentration and relatively normal PTH levels are the results of the Casr mutation in heterozygote or homozygote.

However, previous studies including linkage analyses suggested that the Casr mutations do not explain all the cases of FHH. We examined a unique Japanese FHH family in which the proband and her
mother showed FHH symptoms and had many calcium deposits on their skin. We analyzed the DNA sequences of their Casr genes.

Materials and Methods

Case report

The proband was a girl 5 year and 4 months old as of July 1997 [2]. She was born of non-consanguineous parents. Her high level of thyroid-stimulating hormone (TSH) was revealed at neonatal screening, and she was admitted to the hospital at 44 days old. A high serum TSH, low serum free thyroxine, and high thyroglobulin level were revealed. The size and site of the thyroid was confirmed to be normal both by computed tomography scan (CT scan) and ultrasonography, but the thyroid gland was not detected by Tc-99m scintigraphy. These results showed that the proband’s congenital hypothyroidism was due to an iodine transport defect (ITD). She was then medicated with Levothyroxine. Her parents were not medicated with Levothyroxine, and considered to be euthyroid. At 1-year and 1-month old, many nodules on her abdomen and both sides of the thighs were noted. A skin biopsy was carried out and the nodules were found to be metastatic calcinosis cutis. Similar nodules existed on both hands of her mother. The proband’s father did not have any such nodules. The parathyroid function, and serum calcium, urinary calcium and creatinine levels were examined in the proband and her parents (Table 1). The high serum calcium levels, non-suppressive levels of PTH, low urinary calcium/creatinine ratio, and the calcium-to-creatinine clearance ratios showed that the proband and her mother had familial hypocalciuric hypercalcemia. No renal stones or calcium deposits in the proband’s brain were detected by ultrasonography and CT scan, respectively.

Clinical laboratory assays

Blood and urine samples were collected from the proband and her parents. Second voiding urine was collected when they were visiting a hospital. The serum and urine concentrations of total calcium, creatinine, and inorganic phosphorus were determined with an automated clinical chemistry analyzer. The plasma C-terminal PTH levels (Eiken, normal range < 0.5 ng/ml), was quantified by radioimmunoassay, and intact PTH (Allegro, normal range: 15–50 pg/ml) by immunoradiometric assay.

Experimental subjects

Peripheral blood samples from the proband’s family were obtained. Written informed consent was obtained from all subjects, including the normal control subjects described below.

For the determination of the frequency of polymorphic alleles, the genomic DNA of 44 normal control subjects was obtained. The participants were healthy students attending a high school in Okayama Prefecture in Japan. They agreed to participate in the retrospective study for the analysis of various polymorphisms. The study was approved by the ethics board of our institution.

Polymerase chain reaction (PCR) of genomic DNA and sequence analysis

Genomic DNA was extracted from peripheral blood using the QIAamp Blood kit (Qiagen, Chatsworth, CA). For the identification of the regions causing the disorder, 50 ng of genomic DNA was amplified using a PCR and 12 pairs of oligonucleotide primers spanning the entire 3,234 bp coding sequence of the Casr as previously described [3, 4]. Each primer (10 pmol of each) was used for PCR in

Table 1. Laboratory data of the proband and mother in an FHH family

<table>
<thead>
<tr>
<th></th>
<th>Proband</th>
<th>Mother</th>
<th>Father</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Calcium (mg/dl)</td>
<td>11.3</td>
<td>10.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Serum Phosphorous (mg/dl)</td>
<td>6.0</td>
<td>3.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>0.54</td>
<td>0.78</td>
<td>0.59</td>
</tr>
<tr>
<td>C-terminal PTH (ng/ml)</td>
<td>0.5</td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>Intact PTH (pg/ml)</td>
<td>290</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Urinary Ca/Cr</td>
<td>0.015</td>
<td>0.003</td>
<td>0.298</td>
</tr>
<tr>
<td>CCa/CCr</td>
<td>0.0007</td>
<td>0.0002</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Normal values are given below.

C-terminal PTH (ng/ml): Eiken, normal range < 0.5
Intact PTH (pg/ml): Allegro, normal range 15–50
Urinary Ca/Cr: calcium to creatinine ratio
CCa/CCr: urinary calcium-to-creatinine clearance
—: not tested
a 50 μl reaction mixture containing 10 mmol/L Tris-
HCl (pH 8.4), 1.5 mmol/L MgCl₂, 50 mmol/L KCl,
0.2 mmol/L deoxy-NTPs, and 1 U AmpliTaq poly-
merase (Perkin-Elmer, Foster City, CA). After an
initial denaturing at 95°C for 5 min, 40 cycles of PCR
amplification were carried out in a thermal cycler
(Perkin-Elmer GeneAmp PCR system 2400) with
the following protocol: 94°C for 30 sec, 63°C for
30 sec, and 72°C for 30 sec. After amplification,
the products were purified by spin dialysis using a QIA-
quick PCR Purification kit (Qiagen) for direct double
strand DNA sequencing. An automated DNA se-
quencing analysis with dichlororhodamine dye ter-
minalers (ABI PRISM dRhodamine Terminator Cy-
cle Sequencing Ready Reaction Kit, Perkin-Elmer)
was performed according to the manufacturer's in-
structions (310 Genetic Analyzer, Applied Systems,
Foster City, CA).

RNA extraction and reverse transcriptase (RT)-
PCR

Total RNA was extracted from thyroid tissue,
parathyroid tissue, kidney, and lymphocytes trans-
formed by EB virus, then subjected to the acid
guanidine-phenol-chloroform treatment. The
thyroid and parathyroid tissues were obtained from
a patient with secondary hyperparathyroidism of
chronic renal failure when a total parathyroidectomy
was carried out. The kidney tissue was obtained
from a patient with asymptomatic proteinuria diag-
nosed by renal biopsy. Lymphocytes from a patient
with an unrelated disorder were transformed by EB
virus.

Five μg of RNA was converted into complementa-
ry DNA using a RT-PCR kit (Stratagene, La Jolla,
CA). PCR was performed using the exonic primer
pair 4EF and 6AR according to the PCR program
described above.

Detection of a 30bp polymorphic insertion at
codon 536

After the RT-PCR of region 4EF and 6AR was
performed, a second PCR was performed using Casri
501 as the forward primer and Casri 602 as the
reverse primer (Casri501 5'-TTTAAGGAAGTCG-
GGATT-3', Casri602 5'- TCGGCTGCAGTTGGAGA-
GAAG-3') (Fig. 1). A polymorphic insertion with a
size difference of 30 bp was detected by electropho-
resis on a 4% agarose gel and sequencing.

Statistical analysis

The relationship between the serum calcium con-
centration and genotype of codon 990 was examined
by an analysis of variance (ANOVA).

Results

DNA sequence analysis

No mutation in the coding region of the Casr gene was found in the proband or her mother. The only difference between the proband/her mother and the proband's healthy father was at the first position of codon 990, which was reported to be a polymorphism site [5]. The father was AGG (R) homozygous at codon 990, while the proband and her mother were AGG (R)/GGG (G) heterozygous at codon 990.

Polymorphic frequency

Whether these polymorphic sites relate to the function of the Casr has not been established. We investigated the frequency of polymorphism at codon 990 of the Casr gene. The frequencies and involvement of the other polymorphic sites (codon 536: 30 bp polymorphic insertion, codon 926: CAG, CGG, codon 986: GCC, TCC, codon 1101: CAG, GAG) were also investigated. Genomic DNA from normal subjects was used for a PCR. The products were confirmed by electrophoresis on agarose gels, then purified for the sequence analysis. At codon 990, the prevalence of genotypes AGG/AGG, GGG/GGG, and AGG/GGG was 0.27, 0.41, and 0.32, respectively (Table 2). A polymorphic insertion in codon 536 was confirmed in all the PCR products including the FHH family. We could not confirm the polymorphism of codon 926 in Japanese subjects, including those of the present subjects. At codon 986 genotypes GCC(A)/GCC(A), TCC(S)/TCC(S), and GCC/TCC (A/S) were distributed in the ratio of 0.977, 0, and 0.023, respectively. At codon 1101, the ratios of the genotypes CAG(Q)/CAG (Q), GAG(E)/GAG (E), and CAG/GAG (Q/E) were 0.977, 0, and 0.023, respectively. We could not confirm the prevalence.

Detection of a 30bp insertion at codon 536

At codon 536, the results of electrophoresis showed that all of the amplified PCR products of genomic DNA had a 30 bp polymorphic insertion. This insertion had a splice donor consensus sequence. We therefore searched for splicing variants. Total RNA extracted from lymphocytes transformed by EB

| Table 2. Frequency of four polymorphic sites |
|---|---|---|
| codon 926 & Q & Q/R & R |
| & 0 & 0 & 1 |
| codon 986 & A & A/S & S |
| & 0.977 & 0.023 & 0 |
| codon 990 & G & G/R & R |
| & 0.42 & 0.31 & 0.27 |
| codon 1011 & Q & Q/E & E |
| & 0.977 & 0.023 & 0 |

At codon 926, one allele, CGG(R), was seen in all subjects.
At codon 990, the three alleles were each present at the ratio of about 30%.
At codon 926, codon 986, codon 1011 we could not confirm the significant prevalence rate.

Fig. 2. Splicing of 30bp insertion at codon 536.
All of the PCR products produced 101 bp fragments, which did not contain the 30 bp polymorphic insertion. No splicing variant was detected. The arrow (→) shows 118 bp marker PhiX174/ HaeIII.
Lanes 1. Lymphocytes transformed by EB virus
2. Thyroid
3. Parathyroid
4. Kidney
Familial hypocalciuric hypercalcemia (FHH) was first reported by Foley et al. as familial benign hypercalcemia [6]. The case was a boy who presented with persistent hypercalcemia despite parathyroidectomy. In a study of the families of patients with FHH and primary parathyroid hyperplasia, FHH was characterized by moderate hypercalcemia (\(<12 \text{ mg/dl}\)) and inappropriately low calcium excretion (a calcium to creatinine clearance ratio of \(<0.01\)) [7–10]. FHH could then be distinguished from primary hyperparathyroidism. In previous linkage studies, human chromosome 3q, 19p13.3, 19q13 were mentioned as the genes responsible for FHH [11–13]. By fluorescence in situ hybridization Janicic et al. [14] mapped the Casr gene to 3q13.3-q21. After the Casr gene cDNA was cloned, heterozygous mutations of the Casr gene were reported among cases of FHH.

Because the proband and her mother had symptoms of FHH, we hypothesized that the Casr mutation caused the symptoms of FHH. We investigated the Casr gene of the proband and her mother, but found no mutation in the coding sequence. The only amino acid that was different from her healthy father was at codon 990, known as a polymorphic site. The proband and her mother were heterozygous for allele GGG (G) and AGG (R), while the father was a homozygote for allele AGG at codon 990.

The relevance of polygenic disorders and polymorphisms of genes has been reported for many diseases. For example, polymorphism of the angiotensin-converting enzyme (ACE) gene is known to be associated with the risk of myocardial infarction [15]. The Casr gene was reported to have polymorphic sites at codon 536, codon 926, and codon 990 by Garrett et al. [5], and at codon 986, codon 1101 by Heath et al. [16]. Four of the five polymorphic sites, codon 926, 986, 990, and 1101, are one base permutations of a nucleotide in the Casr gene, and translate into other amino acids. These changes might have some influence on the Casr-PTH-regulated calcium homeostasis. The first possibility is that the transition of an amino acid in each polymorphism changes the receptor function. The second possibility is that the haplotype produced by a combination of each polymorphism alters the function of the Casr.

We found that polymorphisms of codon 926, 986, and 1101 are rare in Japanese and we could not assess the significance of the relationship between these genotypes and the serum calcium concentration [17]. At codon 990, the three genotypes were distributed in the ratio of about 30%. Among the three genotypes, we found no significant difference in serum calcium.

### Table 3. Relationship between three genotypes at codon 990 and the serum calcium concentration

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Calcium concentration (mg/dl)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGG</td>
<td>12</td>
<td>9.384</td>
<td>0.183</td>
</tr>
<tr>
<td>AGG/GGG</td>
<td>14</td>
<td>9.422</td>
<td>0.159</td>
</tr>
<tr>
<td>GGG</td>
<td>18</td>
<td>9.422</td>
<td>0.160</td>
</tr>
</tbody>
</table>

We examined the relationship between the three genotypes at codon 990 and the serum calcium concentrations of 44 subjects by ANOVA. No significant difference was revealed (p=0.4355).
level among normal subjects. Thus, this polymorphism did not have a significant influence on the homeostasis of the serum calcium level.

At codon 536, a 30 bp insertion was confirmed in all of the amplified PCR products of genomic DNA from normal subjects. We investigated the non-splicing products from RT-PCR. Alternative splicing might occur in target organs, and regulate the function of the Casr. Total RNA was extracted from thyroid, parathyroid, kidney, and lymphocytes. We found no insertion in the RT-PCR products from these tissues. The possibility of alternative splicing in target organs was excluded. Garrett et al. mentioned that 30 bp insertion of cDNA was produced by miscloning. Our result of RT-PCR supported this. These results exclude the two possibilities for polymorphisms of the Casr. Thus, in our case it was revealed that the Casr gene is not the cause of FHH.

The proband was administered Levothyroxine for her congenital hypothyroidism due to ITD. The human sodium/iodine symporter (NIS) gene, a member of the sodium-dependent solute symporter family, was recently cloned and mapped to human chromosome 19p13.2–p12, and thought to be a common cause of ITD. Fujiwara et al. [18, 19] investigated five cases of hypothyroidism including the present proband. They confirmed the homozygous T354P mutation of the NIS gene to be present in all patients but the proband. They did not investigate other mutations of the NIS gene in the proband. In this case the patient's parents were not medicated with Levothyroxine, and were considered to be euthyroid state. The possibility of a compound heterozygous mutation was not completely excluded.

There are two types of FHH which do not involve the Casr gene. One of the variants is called hypocalciuric hypercalcemia type II, and has been mapped to 19p13.3, which is near to the NIS gene locus. We assume that there is a mutation at another position in the NIS gene, and that the cause of FHH and ITD are related.

The proband and her mother had early onset of calcium deposits on the skin diagnosed as metastatic calcinosis cutis. Although in FHH cases ectopic calcifications are seldom seen, a few cases with chondrocalcinosis and basal ganglia calcification have been reported [20, 21]. Most of these involved older people, and a higher prevalence of chondrocalcinosis was seen than would be expected in the normal population. Yet involvement of the Casr gene mutation has been reported. To date calcium deposits on the skin as in our case had not been reported. Usually metastatic calcinosis cutis is observed in patients with hyperphosphatemia in renal failure and hypoparathyroidism. In the present case we could not find any evidence of renal dysfunction or hyperphosphatemia. We assumed that the skin lesions were independent of the Casr gene. It should be clarified whether previous cases with ectopic calcification really have mutations of the Casr gene.

In conclusion, we examined a unique FHH family whose members had no mutation of the Casr gene.

Aknowledgments

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References

FAMILIAL HYPOCALCIURIC HYPERCALCEMIA WITHOUT MUTATION


