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Identification of a novel large *CASR* deletion in a patient with familial hypocalciuric hypercalcemia

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Summary

Familial hypocalciuric hypercalcemia type I is an autosomal dominant disorder caused by heterozygous loss-of-function mutations in the *CASR* gene and is characterized by moderately elevated serum calcium concentrations, low urinary calcium excretion and inappropriately normal or mildly elevated parathyroid hormone (PTH) concentrations. We performed a clinical and genetic characterization of one patient suspected of familial hypocalciuric hypercalcemia type I. Patient presented persistent hypercalcemia with normal PTH and 25-hydroxyvitamin D levels. The *CASR* was screened for mutations by PCR followed by direct Sanger sequencing and, in order to detect large deletions or duplications, multiplex ligation-dependent probe amplification (MLPA) was used. One large deletion of 973 nucleotides in heterozygous state (c.1733-255_2450del) was detected. This is the first large deletion detected by the MLPA technique in the *CASR* gene.

Learning points:

- Molecular studies are important to confirm the differential diagnosis of FHH from primary hyperparathyroidism.
- Large deletions or duplications in the *CASR* gene can be detected by the MLPA technique.
- Understanding the functional impact of the mutations is critical for leading pharmacological research and could facilitate the therapy of patients.

Background

Familial hypocalciuric hypercalcemia type I (FHH1, OMIM #145980) is an autosomal dominant disorder transmitted with a high degree of penetrance and characterized by moderately elevated serum calcium concentrations, low urinary calcium excretion and inappropriately normal or mildly elevated parathyroid hormone (PTH) concentrations. This disorder is caused by heterozygous loss-of-function mutations in the *CASR* gene (OMIM +601199) encoding the human calcium-sensing receptor (CaSR).

Multiple disorders have been associated with defects in the CaSR as it plays a central role in the regulation of extracellular calcium homeostasis (1, 2). FHH1, neonatal hyperparathyroidism (NHPT, OMIM #239200) and hypercalciuric hypercalcemia (3) are diseases caused by heterozygous loss-of-function mutations in the *CASR* gene, whereas activating mutations can result in isolated autosomal dominant hypocalcemia (OMIM #601198) or combined with Bartter-like syndrome (OMIM #601198).

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The aim of this study was to perform a clinical and genetic characterization of one patient suspected of familial hypocalciuric hypercalcemia. Patients with mutations of the *CASR* gene may not present such a classic picture of hypercalcemia with hypocalciuria or hypocalcemia with hypercalciuria. Molecular studies are important for confirming the diagnosis and distinguish it from other entities. One large deletion of 973 nucleotides in heterozygous state (c.1733-255_2450del) was detected in our patient. Importantly, as far as we know, this is the first large deletion detected by multiplex ligation-dependent probe amplification (MLPA) technique in the *CASR* gene.

Case presentation

The study was approved by the Ethics Committee for Clinical Research of Euskadi (CEIC-E). The patient is a 56-year-old female of Philippine origin who was referred for evaluation of persistent hypercalcemia in several routine blood laboratory analyses. A history of hypercalcemia was reported in her family as well (her sister showed hypercalcemia and hyperparathyroidism, she does not have a genetic diagnosis yet). Unfortunately, we do not have any information about the parents. Laboratory results showed persistent hypercalcemia (serum calcium of 11.6 mg/dL, reference range 8.5–10.5 and total serum calcium corrected for albumin of 11.7 mg/dL, reference range 8.6–10.2) and serum phosphate at the lower limit of the normal range (2.8 mg/dL, reference range 2.6–4.8), whereas intact PTH (40 pg/mL, reference range 10–65) and 25-hydroxyvitamin D levels (34.4 ng/mL, reference range 8.6–54.8) were within the normal range. The patient exhibited hypocalciuria. Specifically, her urinary calcium was very low (urinary calcium <5 mg/dL, reference range 0–250; urinary creatinine 35 mg/dL, reference range 90–300) despite their high serum calcium levels, and she had a 24-h urine calcium:creatinine clearance ratio (CCCR) <0.01. Therefore, the patient was suspected of FHH and the *CASR* gene genetics analysis was requested. Four years previously, she had developed hyperthyroidism due to Graves–Basedow disease, and she had been treated with antithyroid medications and radioiodine therapy. Furthermore, the patient presented other personal history as hypercholesterolemia, prediabetes and femoral and lumbar osteoporosis (markers of bone remodeling: serum alkaline phosphatase 60 U/L, reference range 46–116 U/L; serum osteocalcin 3.02 ng/mL, reference range 0–22; β-isomerized C-telopeptides 0.12 μg/L, reference range 0–0.84).

Investigation

In order to detect large deletions or duplications in the *CASR* region, a commercially available MLPA kit, SALSA MLPA probemix P177-B2 (MRC Holland, Amsterdam, The Netherlands), was used. This MLPA kit contains 14 MLPA probes specific for the *CASR* gene with amplification products between 130 and 301 nucleotides. In addition, it contains nine reference probes expected to have a normal copy number that we used for the intrasample normalization, and nine internal quality control fragments generating an amplification product smaller than 120 nucleotides.

The exons and flanking intronic sequences of the *CASR* gene (Ensembl identifiers: gene ENSG00000036828; transcript, ENST00000639785.1) were screened for mutations by PCR followed by direct sequencing. We used primers to amplify exons 2–7 together with their splice sites. Furthermore, we designed primers to amplify a large fragment where the large deletion, found by the MLPA technique, is included (Forward: 5′-ACAGGTAAGGGAACCCCTCTT-3′; Reverse: 5′GCCTCCACCACTGATGACAA-3′). Purified amplified products were directly sequenced in both directions with fluorescent dideoxynucleotides (BigDye Terminator v3.1 Cycle Sequencing Kit, Life Technologies). DNA mutation was named according to the Human Genome Variation Society guidelines (www.hgvs.org).

With respect to genotype, one large deletion of 973 nucleotides in heterozygous state (marked in bold in the *CASR* sequence showed in Table 1) was detected by the MLPA technique (Fig. 1). The amplification of exon 7 using specific primers that jointed to the deleted region confirmed the heterozygous state in the patient. Subsequently, to delimit the deletion, we amplified one fragment of 2926 nucleotides by long-PCR and delimited the deletion to 973 nucleotides by sequencing with internal primers. The deletion expands from nucleotide in c.1733-255 position within intron 6 to nucleotide c.2518 within exon 7 (c.1733-255_2450del). This deletion is supposed to generate a protein shorter than WT, as the splice site of exon 7 is affected. Therefore, the mutant CaSR protein probably loses the transmembrane domain and the intracellular carboxyl-terminal tail (amino acids 578 to 1078).

As far as we know, this is the first large deletion detected by MLPA technique in the *CASR* gene.

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Table 1 Gross deletion marked in bold within the *CASR* gene sequence (Ensembl identifiers: gene ENSG00000036828; transcript, ENST00000639785.1).

Exon 6	GTGCCCTTCTCCAACTGCAGCCGAGACTGCCTGGCAGGGACCAGGAAAGGGATCATTGAGGGGGAGCCCACCTGCTGCTTTGAGTGTGTGGAGTGTCCTGATGGGGAGTATAGTGATGAGACAG
Intron 6–7	gtaagggaacccctcttgggcactgtgcagggcttggtccacttcggaggcctggggtcagtgaagcccatggaagggctgggctgagatggtgctttgcacagtttctcatttaacaaaggtatatctgagcatcacaaaatgttgggc atggaggtggaacagaaaaagaatgttagatgttcttttaataccataaaagataacaatacctattacacatctttctcttacttcatattgtcggccaatatatttcctttttgttagagtttagtggccctgcatggacacagttat gtctgctccagactccagagttaataggccatcatagaattgcctgctgggtgcctggcactgaatttgctatcttacttgtgtttttcctgtttctcaggttatttttaggtgggaataataattaaacctatcctatagaattttgag aattagatgattaatattggtaatgcacatggtaagttctagataaatatttgttaaataaaactttggcatattagaagtactctataaatattaactatagttgatattatctcatgttatcctcaccccagtgatggggttattgct gctcccattttataaattaggaaactgaggctctgagaggttaagaagcttgatcaaagtcacatcactagtaagttgtatggccaggattccatccagatctctaactccaaaacccatgctcccttcactgcccaaaactgcatactg cctgggagttagcagagacttagtaaacagttgtcagttgaatgagtctgcagcctccctcatcaaaaagctgtggatgtggagtgaggaaagcacaggtcatgtgcatgggatactctgccttgatctaaaaggaagtctgctctggca tgtctacaaagcccatccatatatccaaaataaatcactctccatgtgagatgtcagatatgttcatttgcctcactagagtagccagtttactgcccatatgtatcccacaacattatgttgtaaacctcaaatattacccaataatat ttaaataaataaatcaggctaagcttgaatcattctgcgaagagttgatggaccatgctagacctctggtgtgcagggcagccacctgtccagcaaggttgatcattccctgccatcagaagatagtgttaagtgaaacaagggc**ctca** **cccatgtcatgtgcaaagggaaaacaaactaacaatagaaccatcagagttacatgaaattaaccatgttattaaacatgtcggggttcagcatatttttgataatgtaaaaatcagaatgatttcatcaaaatggagaaaatatgtagt** **gaccacatccaaaaaactatgtattcccaccaccacatgtacactcacacattttagtctgtgccacacaataactcactcttcactgggacattttacag**
Exon 7	**ATGCCAGTGCCTGTAACAAGTGCCCAGATGACTTCTGGTCCAATGAGAACCACACCTCCTGCATTGCCAAGGAGATCGAGTTTCTGTCGTGGACGGAGCCCTTTGGGATCGCACTCACCCTCTTTG** **CCGTGCTGGGCATTTTCCTGACAGCCTTTGTGCTGGGTGTGTTTATCAAGTTCCGCAACACACCCATTGTCAAGGCCACCAACCGAGAGCTCTCCTACCTCCTCCTCTTCTCCCTGCTCTGCT** **GCTTCTCCAGCTCCCTGTTCTTCATCGGGGAGCCCCAGGACTGGACGTGCCGCCTGCGCCAGCCGGCCTTTGGCATCAGCTTCGTGCTCTGCATCTCATGCATCCTGGTGAAAACCAACCGT** **GTCCTCCTGGTGTTTGAGGCCAAGATCCCCACCAGCTTCCACCGCAAGTGGTGGGGGCTCAACCTGCAGTTCCTGCTGGTTTTCCTCTGCACCTTCATGCAGATTGTCATCTGTGTGATCTGGCT** **CTACACCGCGCCCCCGTCAAGCTACCGCAACCAGGAGCTGGAGGATGAGATCATCTTCATCACGTGCCACGAGGGCTCCCTCATGGCCCTGGGCTTCCTGATCGGCTACACCTGCCTGCTGGCT** **GCCATCTGCTTCTTCTTTGCCTTCAAGTCCCGGAAGCTGCCGGAGAACTTCAATGAAGCCAAGTTCATCACCTTCAGCATGCTCATCTTCTTCATCGT**CTGGATCTCCTTCATTCCAGCCTATGCC AGCACCTATGGCAAGTTTGTCTCTGCCGTAGAGGTGATTGCCATCCTGGCAGCCAGCTTTGGCTTGCTGGCGTGCATCTTCTTCAACAAGATCTACATCATTCTCTTCAAGCCATCCCGCAACA CCATCGAGGAGGTGCGTTGCAGCACCGCAGCTCACGCTTTCAAGGTGGCTGCCCGGGCCACGCTGCGCCGCAGCAACGTCTCCCGCAAGCGGTCCAGCAGCCTTGGAGGCTCCACGGGATC CACCCCCTCCTCCTCCATCAGCAGCAAGAGCAACAGCGAAGACCCATTCCCACAGCCCGAGAGGCAGAAGCAGCAGCAGCCGCTGGCCCTAACCCAGCAAGAGCAGCAGCAGCAGCCCCT GACCCTCCCACAGCAGCAACGATCTCAGCAGCAGCCCAGATGCAAGCAGAAGGTCATCTTTGGCAGCGGCACGGTCACCTTCTCACTGAGCTTTGATGAGCCTCAGAAGAACGCCATGGCCC ACAGGAATTCTACGCACCAGAACTCCCTGGAGGCCCAGAAAAGCAGCGATACGCTGACCCGACACGAGCCATTACTCCCGCTGCAGTGCGGGGAAACGGACTTAGATCTGACCGTCCAGGA AACAGGTCTGCAAGGACCTGTGGGTGGAGACCAGCGGCCAGAGGTGGAGGACCCTGAAGAGTTGTCCCCAGCACTTGTAGTGTCCAGTTCACAGAGCTTTGTCATCAGTGGTGGAGGCAG CACTGTTACAGAAAACGTAGTGAATTCA*TAA*

Discussion

In this study, we have described the first large deletion found in the *CASR* gene by the MLPA technique. Structural analysis of *CASR* gene confirmed that the patient presented a deletion of 973 nucleotides in heterozygous state (c.1733-255_2450del). Patient had been previously diagnosed with FHH, and this deletion affects the splice site of exon 7 in a gene where loss of function is a known mechanism of disease; consequently, this large deletion has very strong evidence of pathogenicity; therefore, it has been classified as PVS1-null variant (4).

It has been hypothesized that some punctual mutations exert a dominant negative effect in heterozygous state, interfering with the WT receptor signal and, as a result, produce a more severe biochemical phenotype (5). As the splice site of exon 7 is included in the deleted region, it is supposed that the mutant protein lacks both the transmembrane and the intracellular domain. Thus, the allele presenting the deletion produces a short protein that is retained in the cytoplasm, as other studies have demonstrated with nonsense mutations (6). We hypothesized that our patient only presents CaSR receptors codified by the allele without the deletion in the membrane, as the mutant protein is probably unable to anchor the cell membrane and is presumed to be non-functional and thus, does not interfere with the WT receptor signal. Therefore, patient presents a less severe phenotype as observed in others patients with nonsense mutations of the *CASR* gene (6). Nevertheless, we recognized the need to conduct functional analysis to confirm the pathogenicity of our novel mutation.

In addition, we found the p.Arg990Gly polymorphism in the allele without the deletion. Some authors have reported that patients with this polymorphism and primary or secondary hyperparathyroidism have lower levels of serum PTH and ionized calcium (7), as shown in our patient. However, the influence of this polymorphism on the patient's phenotype is not clear, as it is very common in East Asia (0.50, according to ExAC, https://exac.broadinstitute.org).

Up to date, only two gross deletions have been described in the literature. The first one was an activating mutation which consisted in a large deletion of 181 amino acids in the carboxyl-terminal tail (Ser895-V1075) (8). Interestingly, patient with this deletion presented autosomal dominant hypocalcemia. Authors suggested that the mutant CaSR exerts a dominant positive effect in the membrane. Moreover, it was observed more defective CaSR in the membrane in heterozygous patients

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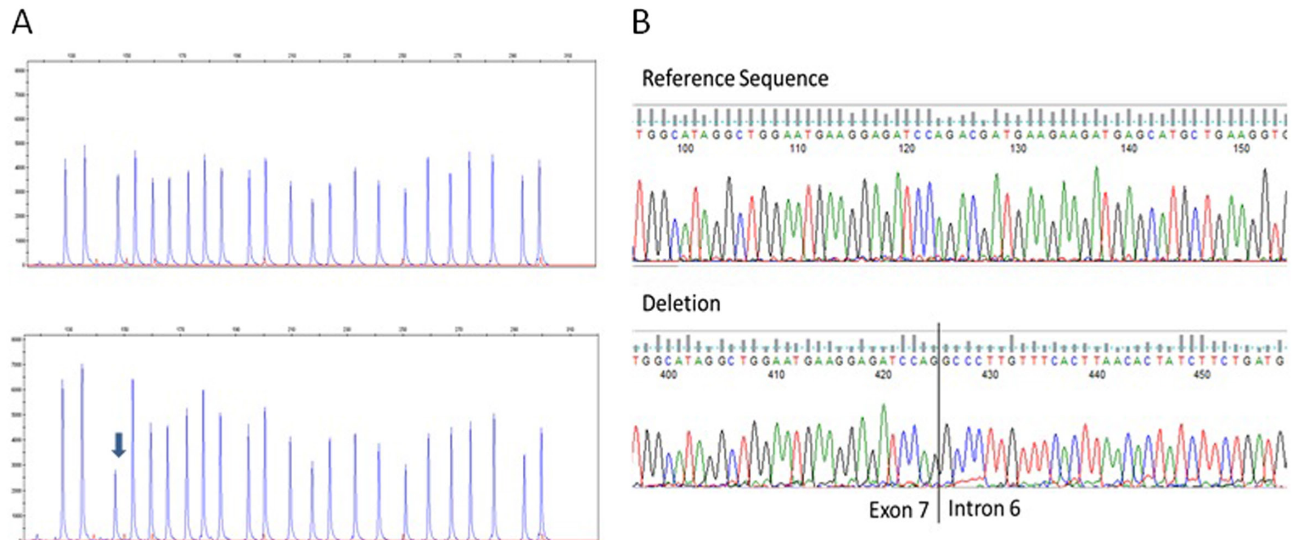

Figure 1
(A) Detection of the *CASR* deletion by the MLPA techniques. MLPA electropherogram for the *CASR* gene from control (upper panel) and patient (lower panel). Each peak represents the exons for the *CASR* gene and nine reference probes. The arrow shows half dose for probe 3 corresponding to the beginning of Exon 7. (B) Novel *CASR* deletion detected by direct sequencing. Figure represents the sequencing chromatograms from control (upper panel) and patient (lower panels). Hemizygous c.1733-255_2450del mutation; vertical line shows the beginning of the deletion.

presenting the deletion. The second deletion consisted in a large deletion of chromosome 3p13.31-22.1 that included 101 known genes, including the *CASR* gene. The individual presented multiple disorders, including hypercalcemia and hypocalciuria (9).

Identification of large deletions by MLPA is remarkably rare in the *CASR* gene. Thus, we only identified one copy number variation in 56 patients (2%) with calcium metabolism disorders using the MLPA technique (data not published). Moreover, a previous study did not identify any copy number variation in a large cohort of 257 patient samples using MLPA (9, 10). In our laboratory, firstly, the exons and flanking intronic sequences of the *CASR* gene are screened for mutations by PCR followed by direct sequencing, and secondly, when the results are negative, the MLPA is performed. This technique is a useful tool to check copy number variations (CNVs). In our experience, the next-generation sequencing shows great sensitivity but little specificity in CNVs detection. Therefore, we propose to first look for mutations by sequencing, should mutation not be detected, MLPA or QMPSF (quantitative multiplex PCR of short fluorescent) analyses must be carried out in order to complete the molecular study.

In conclusion, molecular studies are important to confirm the differential diagnosis of FHH from primary hyperparathyroidism (thus preventing surgery) and other diseases, because of common biochemical features. Understanding the functional impact of the mutations at the cell membrane is critical for leading pharmacological research and could facilitate the therapy of patients. Moreover, performing genetic analysis is essential to detect carriers, because some individuals with mutations remain normocalcemic. The aim of this study was to describe the first gross deletion found using the MLPA technique. Punctual mutations are the most common variants described in the *CASR* gene (according to the Human Gene Mutation Database, 348 mutations). We demonstrated that MLPA is a useful technique for the detection of large deletions or duplications in the *CASR* gene. Therefore, we recommended the use of this technique to complete the molecular study.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this case report.

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Patient consent
The patient provided written, informed consent to participate in this study. The information has been sufficiently anonymized, making it impossible to identify the patient with any certainty.

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Author contribution statement

Writing: A G-C, L M and L C. Study design: A G-C, L M, S A, S G and L C. Study conduct: A G-C, L M, G P d N, A A and L C. Data collection: S A, A A. Data analysis: A G-C and G P d N. Data interpretation: A G-C, G P d N, I M d L, I U and R M. Revising manuscript content: S A, I M d L, S G and L C.

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