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Mutations in the Human Ca^{2+}-Sensing-Receptor Gene That Cause Familial Hypocalciuric Hypercalcemia

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Summary

We report five novel mutations in the human Ca^{2+}-sensing-receptor gene that cause familial hypocalciuric hypercalcemia (FHH) or neonatal severe hyperparathyroidism. Each gene defect is a missense mutation (^{228}Arg→Gln, ^{139}Thr→Met, ^{144}Gly→Glu, ^{63}Arg→Met, and ^{67}Arg→Cys) that encodes a nonconservative amino acid alteration. These mutations are each predicted to be in the Ca^{2+}-sensing receptor's large extracellular domain. In three families with FHH linked to the Ca^{2+}-sensing-receptor gene on chromosome 3 and in unrelated individuals probands with FHH, mutations were not detected in protein-coding sequences. On the basis of these data and previous analyses, we suggest that there are a wide range of mutations that cause FHH. Mutations that perturb the structure and function of the extracellular or transmembrane domains of the receptor and those that affect noncoding sequences of the Ca^{2+}-sensing-receptor gene can cause FHH.

Introduction

The human Ca^{2+}-sensing receptor plays a critical role in determining the level of extracellular Ca^{2+} (Brown et al. 1993; Pollak et al. 1993). This receptor is expressed in the parathyroid gland and kidney, where interaction with Ca^{2+} inhibits release of parathyroid hormone (PTH) and modulates renal reabsorption of Ca^{2+}, respectively, by unknown mechanisms. The proposed topology of the bovine Ca^{2+}-sensing receptor (Brown et al. 1993) is that of a large N-terminal extracellular domain and seven membrane-spanning regions, similar to that of the metabotropic glutamate and glycoprotein hormone receptors (Masu et al. 1991). The Ca^{2+}-sensing receptor's extracellular domain contains regions with a high density of acidic amino acids that are comparable to ion-binding sites in low-affinity Ca^{2+}-binding proteins. The seven membrane-spanning regions and intracellular loops are believed to be important for signal transduction, presumably by coupling to G proteins.

Familial hypocalciuric hypercalcemia (FHH; also known as familial benign hypercalcemia) and neonatal severe hyperparathyroidism (NSHPT) can be caused by mutations in the Ca^{2+}-sensing-receptor gene (Pollak et al. 1993). FHH is generally an asymptomatic disorder inherited as an autosomal dominant trait with ~100% penetrance in childhood. Affected individuals exhibit mild or modest hypercalcemia, relative hypocalciuria, and inappropriately normal PTH levels (reviewed in Marx et al. 1981; Attie et al. 1983; Law and Heath 1985). In contrast, NSHPT is a rare life-threatening disorder characterized by very high serum Ca^{2+} concentrations (sometimes >20 mg/dl), skeletal demineralization, and parathyroid hyperplasia. In some instances NSHPT has been demonstrated to be the homozygous form of FHH (Steinmann et al. 1984; Marx et al. 1992) and is transmitted as an autosomal recessive trait (Pollak et al. 1994*b*).

Elsewhere, we have reported three mutations that cause FHH and NSHPT (Pollak et al. 1993). In two families the mutation is located in the extracellular domain; in another family the defect is in the third intracellular loop of the receptor. To further elucidate the functional consequences of defects in the Ca^{2+}-sensing-receptor gene encoded on chromosome 3, we studied additional families with these clinical conditions. We report five novel missense mutations in the extracellular domain of the Ca^{2+}-sensing-receptor gene in probands from five FHH families. A mutation was not identified in three probands from families with FHH linked to chromosome 3. Collectively, these data suggest that mutations in the large extracellular domain are a common

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cause of FHH. We also speculate that gene defects that perturb expression of the Ca^{2+}-sensing receptor can also produce this disorder.

Material and Methods

Collection of Samples

Informed consent was obtained from all participants or their guardians, in accordance with standards established by local institutional review boards for the protection of human subjects from research risks. DNA from members of 22 unrelated families or individuals with definite or possible FHH or NSHPT was studied. The diagnosis of FHH was based on the finding of asymptomatic hypercalcemia accompanied by normal levels of PTH. The diagnosis of NSHPT was made on the basis of marked hypercalcemia presenting shortly after birth and marked elevation of serum PTH levels. Total serum and urinary calcium concentrations were measured by colorimetric assays. Detailed clinical and laboratory descriptions of several of these families have been reported elsewhere.

Amplification of Genomic DNA and RNase Protection Assays

RNase protection assays to detect mutations in the Ca^{2+}-sensing-receptor gene, described elsewhere (Pollak et al. 1993; Watkins et al. 1994), involved the following steps: Genomic DNA was isolated from either peripheral lymphocytes or Epstein-Barr virus–transformed cell lines (Holcombe et al. 1987; Jacobs and Celeste 1989). Ten segments (100–900 bp each) of the Ca^{2+}-sensing-receptor gene encompassing the entire coding region were amplified by PCR (Pollak et al. 1993). PCR-amplified proband DNA was hybridized to ^{32}P-labeled RNA probes. The resulting RNA-DNA hybrid was digested with RNase A (Sigma). The digestion was stopped by the addition of SDS and proteinase K and analyzed on a 6% denaturing polyacrylamide gel. Riboprobes 1–5 were derived from the restriction fragments of genomic clones and contained splice donor/acceptor sequences; riboprobes 6–8 were derived from subclones of a kidney cDNA. To increase the probability of identifying all mutations, both sense and antisense riboprobes were used. Amplified DNA samples yielding abnormal RNase cleavage patterns were reanalyzed with new DNA isolates to exclude artifacts arising from the PCR.

Sequencing of PCR Products

To demonstrate the presence of these mutations in the Ca^{2+}-sensing-receptor gene in genomic DNA samples, the relevant PCR product was either sequenced directly (using the Stratagene Cyclist kit) or subcloned into the *Bam*HI/*Eco*RI site of the Bluescript II SK vector (Stratagene) and sequenced (Sequenase kit, USB).

To exclude possible sequence differences that arose during PCR amplification or cloning, the samples were reamplified and assayed for the mutation by one of two techniques. The mutations detected in families I (^{63}Arg→Met) and Q (^{67}Arg→Cys) are predicted to change a restriction site and were confirmed by digests with relevant restriction enzymes. Mutations found in families A (^{228}Arg→Gln), C (^{139}Thr→Met), and D (^{144}Gly→Glu) were confirmed by oligo-specific hybridization (Jacobs and Celeste 1989).

LOD Score Calculations

LOD scores were calculated using the computer program MLINK (Ott 1991) assuming recombination distance 0, 100% penetrance, and FHH gene frequency or mutant allele frequency of .01.

Results

Clinical Data

Affected individuals from 20 families with FHH and/or NSHPT were studied. Both affected and unaffected individuals were available for study from eight of these families. Their pedigrees and clinical evaluations have been reported elsewhere (families A, B, C, and D in Chou et al. 1992; family I in Marx et al. 1985; family P in Menko et al 1983; and families Q and S in Pollak et al. 1994*b*). Only one proband was available for study in the remaining 12 FHH families. The clinical features of FHH in these probands were not significantly different from those described previously (Marx et al. 1981; Law and Heath 1985).

RNase Protection Analysis of the Ca^{2+}-Sensing-Receptor Gene in FHH/NSHPT Probands

RNase protection assays were used to screen the six exons of the Ca^{2+}-sensing receptor, as described elsewhere (Pollak et al. 1993). Riboprobes 1–5 were derived from subcloned restriction fragments of genomic clones; riboprobes 6–10 were derived from subclones of a kidney cDNA clone (fig. 1). To increase the probability of identifying all point mutations or small alterations, both sense and antisense riboprobes were used.

Genomic DNA from two affected members, each, of eight kindreds (families A, B, C, D, I, Q, and S) and 12 unrelated FHH probands was amplified using PCR, followed by RNase protection assay (see Material and Methods). Sequence variations between the riboprobes (derived from the wild-type Ca^{2+}-sensing-receptor gene) and DNA samples from these individuals were recognized by abnormal-sized cleavage products (fig. 1). DNAs bearing single point mutations generally produce two abnormal RNA fragments resulting from cleavage of the riboprobe. Abnormal cleavage bands were found in exon 2 (705–1,004 bp) from probands of families C, D, I, and Q and in exon 3 (1,004–1,532 bp) from probands of family A.

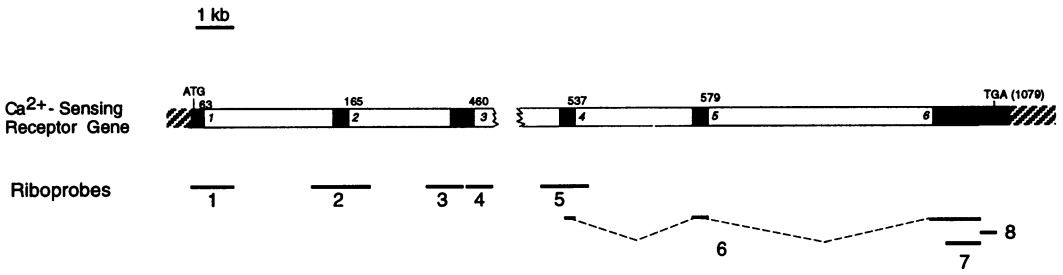

Figure 1 Schematic map of the Ca^{2+}-sensing-receptor gene, indicating the location and size of each exon (numbered 1–6). Riboprobes (1–8) used in RNase protection assays are depicted beneath the map. The location of eight mutations found in FHH/NSHPT families (identified by capital letters) are shown.

Point Mutations in the Ca^{2+}-Sensing Receptor

DNAs yielding aberrant RNase protection patterns were characterized by nucleotide sequence analysis of subcloned PCR products (see Material and Methods). The nucleotide sequences of these variants were independently confirmed either by hybridization to oligonucleotides specific for the nucleotide change or by digestion with a restriction enzyme whose recognition site was altered by the sequence change (data not shown). Five novel sequence changes were identified (table 1). Each of the observed nucleotide changes are predicted to cause amino acid substitutions in the Ca^{2+}-sensing receptor.

To demonstrate that these sequence variants are not common polymorphisms and are associated with disease, two experiments were performed. First, we examined DNAs from 50 normal individuals for the presence of these mutations. None of the 100 normal chromosomes represented in these individuals had any of the missense mutations (data not shown). Second, DNAs from affected and unaffected family members were tested for the presence of the mutation initially identified in probands. In each family, the mutation was present in all DNA samples derived from affected individuals and was absent from samples derived from all unaffected family members. The likelihoods that the disease and the mutation are co-inherited in each of the five families are indicated in table 1.

RNase protection assays of probands from three families (B, P, and S) and 12 unrelated probands failed to identify a mutation. To determine whether genetic heterogeneity could account for this finding, linkage studies were performed in the three families. FHH/NSHPT was linked to the Ca^{2+}-sensing-receptor gene on chromosome 3 in each of these kindreds. By using the polymorphic locus D3S1303 (Weissenbach et al. 1992), a maximum two-point lod score of 4.52 was achieved in FHH family B, 4.5 was achieved in FHH family P, and 1.89 was achieved in NSHPT family S. To determine whether mutations in the splice donor or acceptor sequences might account for the disorders in these families, small regions of flanking intron sequences were analyzed (data not shown); no abnormalities were identified.

Table 1

Mutations in the Ca^{2+}-Sensing-Receptor Gene

Family	Exon	Codon Change	Amino Acid Change	LOD (at $\theta = 0$)
A	3	GCA→CAA	Arg228Gln	9.29
C	2	ACG→ATG	Thr139Met	9.27
D	2	GGA→GAA	Gly144Glu	3.10
E[a]	3	GAG→AAG	Glu298Lys	2.70
I	2	AGG→ATG	Arg63Met	3.82
J[a]	6	CGG→TGG	Arg796Trp	2.41
N[a]	3	CGA→CAA	Arg186Gln	4.47
Q	2	CGT→TGT	Arg67Cys	1.79

[a] Described elsewhere (Pollak et al. 1993).

Discussion

We describe five sequence variations, found in the Ca^{2+}-sensing receptor, that cause FHH and NSHPT. All five sequence variations share three features. First, they each alter a single nucleotide that changes the encoded amino acid (^{228}Arg→Gln, ^{139}Thr→Met, ^{144}Gly→Glu, ^{63}Arg→Met, and ^{67}Arg→Cys). Second, they are not found in >100 normal chromosomes. Third, they are co-inherited with disease in the affected individuals' families. We conclude that all five sequence variations cause FHH.

The Ca^{2+}-sensing receptor is translated from a 5.4-kb mRNA that is encoded in >20 kb of genomic DNA (Brown et al. 1993). On the basis of homology with previously described receptors, the Ca^{2+}-sensing receptor appears to be a member of the G-protein-coupled receptor superfamily (fig. 2). The consequences of mutations in this receptor may be indirect and alter polypeptide folding, receptor processing, or stability. However, the distribution of FHH/NSHPT mutations within this gene suggests that specific structure-function relation-

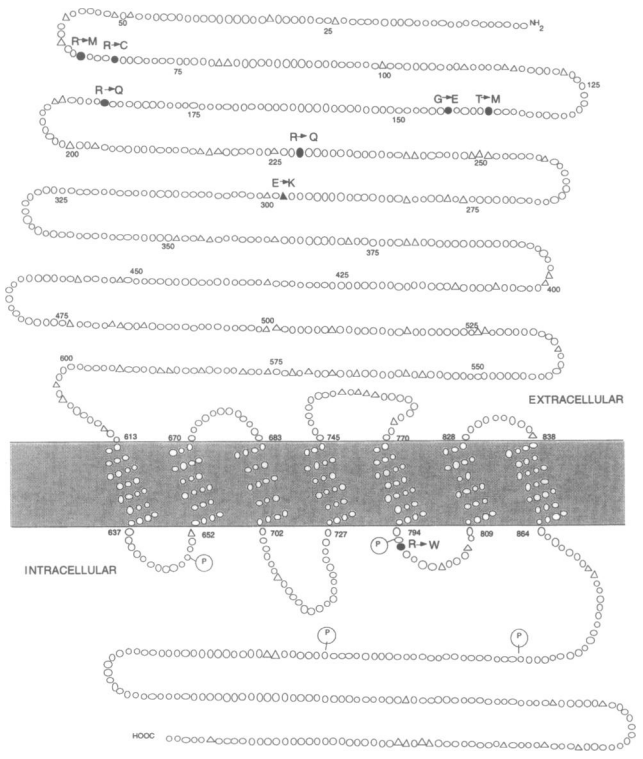

Figure 2 Locations of FHH-causing amino substitutions in the Ca^{2+}-sensing-receptor gene. Acidic amino acid residues are denoted by triangles; others are denoted by circles. Potential protein kinase C phosphorylation sites are shown.

ships are perturbed by these gene defects. To date, 13 mutations have been reported in exons 2, 3, and 6 (Pollak et al. 1993, 1994*b*; Pearce et al. 1994; and data in the present paper). Mutations in exons 2 and 3, which are postulated to encode the large extracellular domain of this protein, produce two different phenotypes. While most of the mutations identified in this region cause FHH and NSHPT, one mutation, ^{128}Glu→Ala, results in autosomal dominant hypocalcemia (Pollak et al. 1994*a*). The divergent phenotypes associated with these mutations suggest that the extracellular domain is critical for determining the receptor's affinity or interaction with Ca^{2+}; mutations within this region appear to reset (up or down) ion binding and receptor activation. Four mutations have also been identified within exon 6 (Pollak et al. 1993; Pearce et al. 1994), which encodes the membrane-spanning segments, intracellular loops, and carboxy-terminal tail of the receptor. It seems likely that FHH produced by these mutations results from perturbation of signal transduction following ligand binding.

It is noteworthy that mutations were not identified in coding sequences of the Ca^{2+}-sensing-receptor gene, in splice donor or acceptor sequences (data not shown) in probands from three families with FHH that is linked to chromosome 3q, or from 12 individual FHH probands. While genetic heterogeneity may explain the absence of mutation detection in the latter group, several studies (Heath et al. 1993; Pollak et al. 1994*b*) have demonstrated that most FHH is due to a gene defect at the 3q locus. RNase protection assays were employed to detect mutations. Because the sensitivity of mutation detection by assays that employ both sense and antisense riboprobes is quite high (Kaufman et al. 1990), it is unlikely that these analyses failed to identify coding sequence mutations. Our previous studies (MacRae et al. 1994) suggested that >95% of all missense mutations are detected using these methods. The riboprobes used in these studies (detailed in Pollak et al. 1993) contained all intron and exon boundaries except those flanking exons 5 and 6. Therefore, most mutations that interrupted splice signals should have been identified. We presume that the gene defects in some FHH families are located in the noncoding sequences but not in the conserved splice signals immediately 5′ and 3′ of each exon. These mutations may cause subtle alterations in the regulated expression of the Ca^{2+}-sensing-receptor gene or may have more substantial effects on gene transcription, as has been recently described in the factor VIII gene (Naylor et al. 1993). Regardless, it appears that mutations that alter receptor number and/or density can also cause the FHH phenotype.

The effect of different missense mutations on the hypercalcemic phenotype is difficult to ascertain from these studies. As multiple different clinical laboratories were used to measure serum Ca^{2+} levels, we are unable to draw conclusions regarding the effect of a specific mutation on serum Ca^{2+} concentration. However, affected individuals in family I (^{63}Arg→Met) had quite mild hypercalcemia. In contrast, one family I member who was homozygous for this mutation had NSHPT with marked hypercalcemia (Marx et al. 1985). We conclude that the potential of a mutation to cause life-threatening disease in the homozygous state cannot be predicted from the heterozygous phenotype.

We suggest that many mutations within the Ca^{2+}-sensing receptor cause FHH, NSHPT, or familial hypocalcemia. Of the 13 mutations identified to date, each are unique, implying little if any founder effect in these disorders. In view of the mild symptoms associated with FHH and the absence of any effect on the reproductive fitness in affected individuals, it also seems possible that individuals with mild asymptomatic hyperparathyroidism and/or borderline hypercalcemia may actually have mutations (or polymorphisms) in their Ca^{2+}-sensing-receptor genes.

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