Calcium-sensing receptor mutations and denaturing high performance liquid chromatography

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Abstract

The calcium-sensing receptor (CASR), a plasma membrane G-protein-coupled receptor, is expressed in parathyroid gland and kidney, and controls systemic calcium homeostasis. Inactivating CASR mutations are associated with familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism, and activating mutations cause autosomal dominant hypocalcemia (ADH). CASR mutation identification plays an important role in the clinical management of mineral metabolism disorders. We describe here a high-throughput method using screening with denaturing high performance liquid chromatography (DHPLC) to initially interrogate 12 amplicons covering translated exons and exon/intron boundaries, followed by sequencing of any amplicon with a modified melting curve relative to wild type, and direct sequencing of a 13th amplicon encoding the COOH-terminal tail to distinguish causative mutations from three common missense single nucleotide polymorphisms. A blinded analysis of 32 positive controls representing mutations throughout the CASR sequence, as well as 22 negative controls, yielded a concordance rate of 100%. We report eight novel and five recurrent FHH mutations, along with six novel and two recurrent ADH mutations. Thus, DHPLC provides a rapid and effective means to screen for CASR mutations.

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Introduction

The calcium-sensing receptor (CASR) is a plasma membrane G-protein-coupled receptor (GPCR) abundantly expressed in the parathyroid gland and kidney tubule where it plays a key role as the ‘calciosstat’, maintaining extracellular fluid calcium concentrations within a narrow normal range (Brown 2007). Activation of CASR by increased ionized calcium leads to decreased PTH secretion and increased calcium excretion. Naturally occurring CASR mutations contribute to perturbed blood calcium levels in several disease states. Hereditary hypercalcemic disorders such as familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT) can be caused by heterozygous and homozygous inactivating mutations in the CASR respectively (Pollak et al. 1993). Similarly, hypocalcemic disorders such as autosomal dominant hypocalcemia (ADH) can be associated with activating mutations in the receptor (Pollak et al. 1994).

The human CASR (MIM #601199) is encoded by six exons (exons 2–7) of the gene (Pollak et al. 1993, Pearce et al. 1995, Heath et al. 1996) located on chromosome 3q13-3q21 (Janicic et al. 1995). Exons 1A and 1B encode alternative 5′ untranslated regions (5′-UTR) (Garrett et al. 1995, Chikatsu et al. 2000, Canaff & Hendy 2002), exon 2 encodes the common 5′-UTR, the ATG initiation codon, the signal peptide sequence, and the beginning of the extracellular domain (ECD); exons 3–6 encode the main part of the ECD; and exon 7 encodes the end of the ECD, the transmembrane domain (TMD), and the intracellular domain (ICD). The human receptor consists of 1078 amino acids, with ~ 600 amino acids in the ECD, 250 in the TMD, and 216 in the cytoplasmic tail.

Over 100 mutations in the CASR have been described in association with FHH and/or NSHPT (Pidasheva et al. 2004). The majority are missense, with others being nonsense, insertion, and deletion/insertion. There are no mutation ‘hot spots’ but the mutations tend to cluster in two regions: the NH2-terminal 300 amino acids of the ECD; and a 340-residue stretch in the transmembrane and ICD (Hendy et al. 2000, see www.casrdb.mcgill.ca). About 100 mutations in the CASR have been described in association with ADH (Pidasheva et al. 2004); most are missense while a
handful in the intracellular tail are deletions. Activating mutations are more tightly clustered in a 150-amino acid stretch in the first NH₂-terminal third of the ECD, and the latter half (~100 amino acids) of the TMD. A few mutations are also found in the intracellular tail (Hendy et al. 2000, see www.casrdb.mcgill.ca).

Various polymorphic variants have been identified in the CASR gene (Yun et al. 2007). Of note, three single nucleotide polymorphisms in exon 7 encode non-conservative amino acid changes (A986S, R990G, and Q1011E) in the COOH-terminal tail of the CASR protein. These polymorphisms have been found to be predictive of serum calcium concentrations in some normal Caucasian populations, either individually or in haplotype combination (Cole et al. 1999, 2001, Scillitani et al. 2004).

Identification of CASR mutations plays an important role in the clinical management of inherited hypercalcemic and hypocalcemic disorders. Positive CASR mutation testing is critical in differentiating FHH cases (in which the mild hypercalcemia is benign) from primary hyperparathyroidism (in which the hypercalcemia may be associated with disturbed bone and mineral metabolism). Thereby, the FHH individual may be spared an unnecessary parathyroidectomy. Assignment of a hypocalcemic individual as having an activating CASR mutation distinct from hypoparathyroidism of other etiologies alerts the physician to the special treatment requirements of such patients related to their over-activated renal CASR and greater risk of nephrocalcinosis or renal stones with vitamin D metabolite supplementation (see Hendy & Cole 2007).

Conventional CASR mutation searches begin with isolation of leukocyte DNA, followed by amplification of all six protein-coding exons and flanking intronic sequences and bidirectional sequencing of all amplicons. Alternative methods, e.g. RNase protection assay (Pollak et al. 1993, 1994) or pre-screening of amplicons by single-strand conformational polymorphism analysis (Pearce et al. 1995), have been reported in some studies but they have not achieved wide application. A newer technique that detects heteroduplexes in PCR amplicons by ion-pair reverse-phase high performance liquid chromatography has been developed (Oefner & Underhill 1998, Xiao & Oefner 2001). Denaturing high-performance liquid chromatography (DHPLC) has proved useful for mutational analysis of genes such as MEN1 (Crépin et al. 2006), RBL1 (Houdayer et al. 2004), ENG and ALK-1 (Lenato et al. 2006), NPHS2/podocin (He et al. 2007), and BRCA1/2 (Gerhardus et al. 2007). We (Hendy et al. 2003) and others (Waller et al. 2004) have successfully utilized the DHPLC method for CASR mutational analysis in a few cases. Here, we present a detailed outline of the development and evaluation of a rapid throughput DHPLC protocol that allows amplicons of wild-type sequence to be screened out and only those showing evidence of heterozygosity selected for sequencing and mutation identification.

Materials and methods

Positive and negative control patient panels

Genomic DNA samples from 32 patients having either FHH/NSHPT or ADH and in whom CASR mutations/sequence variants (positive controls) had been previously identified by conventional (non-DHPLC) methods were subjected in a blinded fashion to DHPLC analysis. A further set of genomic DNA samples from 22 hypercalcemic or hypocalcemic individuals (presumptively FHH or ADH patients) in which no CASR mutations had been identified by conventional (non-DHPLC) analysis were examined by the DHPLC method. In addition, DHPLC analysis of selected samples from a study (Guarnieri et al. 2008) of a cohort of hypercalcemic patients (and controls) seen at the Endocrine Clinic, San Giovanni Rotondo Research Centre (Istituto di Ricovero e Cura a Carattere Scientifico, Casa Sollievo della Sofferenza, Italy) is presented here as representative of the utility of the method. FHH was diagnosed by asymptomatic hypercalcemia with high-normal or slightly elevated serum PTH levels. Diagnosis of NSHPT was based on marked hypercalcemia and serum PTH levels presenting shortly after birth. ADH was diagnosed by the finding of hypocalcemia with low or low-normal serum PTH levels. All subjects gave informed consent and studies were approved by institutional ethical committees.

Nucleic acid extraction and amplification

Genomic DNA was extracted from peripheral leukocytes using standard methods. The CASR protein-coding regions and intron/exon boundaries were PCR amplified as 13 fragments ranging in size from 202 to 455 bp (see Table 1 for specific primer sequences). For some primers, 5’ and 3’ GC clamp sequences were used to confer optimal melting profiles on each amplicon. Three basic GC clamp sequences supplied by Transgenomic (San Jose, CA, USA) (an 8-bp sequence (5'-gcgtcccg-3’), a 10-bp sequence (5'-gccccccgccg-3’), and a 20-bp sequence (5'-gcgccccgccccccgggccccccggc-3’)) were used either as is or shortened to produce an optimal melt profile as predicted by Wavemaker v. 4.1 software. Genomic DNA (2.5 ng/ml) was amplified in 1× PCR buffer (Qiagen), 0.2 mM of each dNTP, 0.5 mM of each primer, and 0.025 U/ml HotStarTaq (Qiagen) in a total volume of 20 µl. The thermal cycles comprised an initial DNA denaturation and HotStarTaq activation at
Table 1  PCR primers and annealing temperature for each amplicon and denaturing high performance liquid chromatography conditions used

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Size (bp)</th>
<th>Primer sequences(^a)</th>
<th>PCR annealing temperature</th>
<th>DHPLC initial %B</th>
<th>Elution temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>305</td>
<td>F: 5’-gcctcccgcgCTCATCGTGTCACTCCCTTG-3’ (5’ UTR)(^b)</td>
<td>60</td>
<td>53</td>
<td>60, 63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TTTGGTGCGACTTCTCC-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3-1</td>
<td>202</td>
<td>F: 5’-gcctcccgcgCCTCCATTTTCTCCTCCT-3’ (intronic)</td>
<td>60</td>
<td>50</td>
<td>59.5, 60.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-AACCCTGACTGCTGCTG-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3-2</td>
<td>264</td>
<td>F: 5’-gcctcccgcgCTCCCTCCTCCTCCTCCG-3’</td>
<td>60</td>
<td>53</td>
<td>60-65</td>
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<tr>
<td></td>
<td></td>
<td>R: 5’-GCCTGCTCCTGATGATG-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4-1</td>
<td>398</td>
<td>F: 5’-gcccgccgTACCTACCTACCTCCTCCT-3’ (intronic)</td>
<td>60</td>
<td>57</td>
<td>60-65, 61-65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-gcctcccgcgCTTGTAGGAGGGGTGCTGTCAG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4-2</td>
<td>377</td>
<td>F: 5’-gcctcccgcgCATGTGTAGAGGGATTTCCCAAAGG-3’</td>
<td>60</td>
<td>56</td>
<td>60-65, 61-65, 63</td>
</tr>
<tr>
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<td>R: 5’-TTGaCTGCCACTCCCTCAAGAG-3’</td>
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</tr>
<tr>
<td>E4-3</td>
<td>367</td>
<td>F: 5’-gcctcccgcgCTTGTAGAGGGATTTCCCAAAGG-3’</td>
<td>60</td>
<td>56</td>
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<td></td>
<td></td>
<td>R: 5’-GCACCCCACTGCTTCCAT-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>343</td>
<td>F: 5’-CAGGGCCAGCTCCTCAAT-3’ (intronic)</td>
<td>60</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-CCTGCTGAGAACCCTCAAGG-3’</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E6</td>
<td>221</td>
<td>F: 5’-gcctcccgcgCTGCGGCCCTCCTACACAC-3’ (intronic)</td>
<td>60</td>
<td>51</td>
<td>62, 63</td>
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<td></td>
<td>R: 5’-gcctcccgcgACAGTGCCCAAGGAGGTTGTC-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7-1</td>
<td>341</td>
<td>F: 5’-gccggccgccgGAGTTGAGGAGTTGACCAAC-3’ (intronic)</td>
<td>60</td>
<td>55</td>
<td>62</td>
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<tr>
<td></td>
<td></td>
<td>R: 5’-AGAACAAGGAGCTGAGAAGAAC-3’ (intronic)</td>
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<tr>
<td>E7-2</td>
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<td>F: 5’-gccggccgccgGAGTTGAGGAGTTGACCAAC-3’ (intronic)</td>
<td>60</td>
<td>54</td>
<td>63-65, 64.5</td>
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<tr>
<td></td>
<td></td>
<td>R: 5’-gcctcccgcgCTGAGGAGGAAAACAACG-3’</td>
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<td></td>
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<tr>
<td>E7-3</td>
<td>414</td>
<td>F: 5’-CTGGCTGTATGAGGCACATGAGT-3’</td>
<td>58</td>
<td>57</td>
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<td></td>
<td></td>
<td>R: 5’-GATGGCAATCTGACCTACCTACG-3’</td>
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<td></td>
<td></td>
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<tr>
<td>E7-4</td>
<td>432</td>
<td>F: 5’-gcctcccgcgGAGTTGAGGAGTTGACCAAC-3’ (intronic)</td>
<td>62</td>
<td>57</td>
<td>62, 65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-gcctcccgcgGAGTTGAGGAGTTGACCAAC-3’ (intronic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7-5</td>
<td>455</td>
<td>F: 5’-CTAAACCCGGCAAGGAGACGCA-3’ (3’ UTR)</td>
<td>60</td>
<td>NA(^c)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TCTCCCTAGCCAGGACAGCTC-3’ (3’ UTR)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)GC clamp sequences are lowercase and bolded.

\(^b\)Sequences are exonic except where indicated otherwise.

\(^c\)Amplicon E7-5 examined by sequence analysis not by DHPLC (see text).

95 °C for 15 min, then 35 cycles of 94 °C for 20 s, annealing at 58-62 °C (see Table 1 for specific temperatures for each amplicon) for 20 s, and elongation at 72 °C for 20-25 s with an increment of 1 s after each cycle, and a final extension at 72 °C for 5 min. Samples were then annealed by initially heating to 95 °C for 5 min and cooling slowly to 30 °C over 30 min to allow heteroduplex formation. The samples were then either directly analyzed or stored at 4 °C before analysis by DHPLC.

**DHPLC analysis**

Heteroduplexes were resolved on a Transgenic WAVE system equipped with a DNAsSep Cartridge column. Five microliters of PCR product were injected and eluted at the partial melting temperature with an incremental acetonitrile gradient of 9% in 4-5 min at a constant flow rate of 0-9 ml/min. The gradient was created by mixing buffer A (0-1 M triethylamine acetate buffer (TEAA), pH 7, 0-1 M Na₂EDTA) with buffer B (25% acetonitrile, 0-1 M TEAA, and 0-1 M Na₂EDTA); the initial concentrations of buffer B were adjusted according to the length of the fragment. After each analysis, the column was stripped with 100% acetonitrile for 0-5 min and regenerated in buffer B at 5% below the initial concentration for 1-5 min. The elution profile was monitored by u.v. absorbance at 260 nm.

**DNA sequencing**

Any fragment (from an experimental sample) that generated a modified DHPLC melting curve compared with the control (from wild type) was further examined by DNA sequencing. Twenty microliters of each PCR product were purified using the QIAquick PCR Purification kit (Qiagen) and eluted in 30 µl of buffer. The PCR products were directly sequenced in sense and antisense directions using the appropriate DHPLC–PCR primer set (but lacking any GC clamp sequences), using the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City; CA, USA) and BigDye Terminator Cycle Sequencing Kit (v.1.1) according to the manufacturer’s protocol. The E7-5 fragment, harboring the three common polymorphisms, A986S, R990G, and Q1011E was analyzed by DNA sequencing.
Bioinformatics

PolyPhen (PHEN – http://genetics.bwh.harvard.edu/pph/) (http://tux.embl-heidelberg.de/ramensky/index/html) predicts the effect of an amino acid substitution on function based on knowledge of the protein’s structure, interactions, and evolution (Sunyaev et al. 2001, Ramensky et al. 2002). The effect of missense CASR variants, classified as either probably damaging, possibly damaging or benign, was predicted from a position-specific independent counts score based on alignment of the sequences of known CASR orthologs in several species as well as of homologous group 3 GPCRs.

SIFT (Sorting Intolerant From Tolerant) (http://blocks.fhcrc.org/sift/SIFT.html) predicts whether a particular amino acid substitution would be tolerated or not tolerated (affect protein function) based on a homology analysis of the protein of interest (CASR) from several different species, as well as related proteins (group 3 GPCRs) (Ng & Henikoff 2003).

Results

DHPLC protocol optimization and validation

The entire CASR protein-coding sequence and the intron–exon boundaries were amplified as 13 fragments and the first 12 amplicons were subjected to DHPLC, while the 13th (E7-5 encoding three common polymorphisms) was subjected to direct sequencing (Fig. 1). The method was validated by the use of genomic DNA samples representing 32 CASR mutations/sequence variants (positive controls) previously identified by conventional (non-DHPLC) methods (Table 2). The positive controls were spread throughout the CASR-coding sequence, and covered most of the melting domains present in each fragment. Figure 1 details the relative position of each of the positive control mutations of the present study along with other reported mutations. Areas of high, medium, and low confidence of sequence variation detection are indicated. High confidence areas are those with optimal melt profiles and where analysis of positive controls, and/or detection of a sequence variant in an experimental sample (Guarnieri et al. 2008), provided confirmation of the capability to detect sequence alterations. Medium confidence areas are those with optimal melt profiles; however, no positive control or experimental samples were available within these areas. Low confidence areas do not have optimal melt profiles and no positive control or experimental samples were available. All sequence variations of the positive control samples were identified under conditions of at least one temperature, with the majority being detected at several analytical temperatures (Table 1).

Optimization of the chromatographic conditions provided clear cut differences in the elution profiles of variant-containing and wild-type amplicons. For example, exon 4-2 amplicons harboring missense changes, T263M and 1283T, or P274S, R285W or F351V, were clearly distinguished from wild type at 63°C or 61.5°C respectively (Figs 2 and 3). These particular mutations were identified in a study of a hypercalcemic cohort at a single endocrine clinic (Guarnieri et al. 2008), and DHPLC chromatographic profiles are included here to demonstrate the utility of the method.

A panel of 22 negative controls comprising DNA samples from 15 hypercalcemic (presumptively FHH) and 7 hypocalcemic (presumptively ADH) patients, in which all protein-coding exons and intron/exon boundaries were of normal sequence by conventional (non-DHPLC) analysis, were confirmed as being of wild-type sequence by the DHPLC method.

Clinical and biochemical data

Of the FHH and ADH mutations represented in the positive control panel some have been previously published by us, some are recurrent (having been identified in other families or individuals), and some are novel (Table 2). The clinical and biochemical data available for these cases can be found as the supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol42/issue4.

Bioinformatics

We applied a bioinformatics analysis to the 16 FHH (inactivating-type) missense mutations in the panel (Table 3). All the amino acids involved (with one exception) are absolutely conserved among species. For all mutations, Polyphen predicted 11 to be damaging and 5 to be benign. SIFT predicted 12 to affect function (not tolerated) and 4 to be tolerated. There was discordance between the two programs for only 2 out of the 16 mutations. For six of the mutants, a previous in vitro analysis had been conducted demonstrating impairment relative to the wild-type CASR. Three (out of the six) were predicted by Polyphen to be damaging, whereas five were predicted by SIFT to affect function (Table 3).

Discussion

Development and evaluation of the DHPLC protocols

While the CASR protein-coding mutations tend to cluster in the first 300 amino acids of the ECD and
the TMD (TMD; amino acids 500–900), a comprehensive mutation-screening method encompassing all protein-coding exons and exon/intron junctions is required. The semi-automated DHPLC method described here provides a rapid and sensitive way of screening all CASR exons to select only those amplicons with a chromatographic profile which departs from wild type for further analysis by sequencing. A blinded...

Figure 1 Panel (A) genomic organization of the CASR that is comprised of eight exons. Untranslated regions, exons 1A and 1B, part of exon 2, and part of exon 7 are in gray, while part of exon 2, exons 3–6, and part of exon 7 that are protein-coding are in black. P1, promoter 1, P2, promoter 2. Panel (B) the 13 amplified CASR fragments that range in size from 202 to 455 bps (fragment sizes with GC clamps are shown in parentheses). Mutations reported in the CASR mutation database (http://www.casrdb.mcgill.ca/) and in the literature are indicated above the line: missense – inactivating (i), activating (a), unknown (u) – silent mutations (s), insertion/deletion (d, i), and SNPs (s). Mutations found through our in-house mutation screening are indicated as missense (m) or deletion (d). Positive controls are presented as (c). Note the large Alu insertion in E7-4 at codon 876 (red arrow) and the large deletion of codons 895–1075 overlapping E7-4 and E7-5 (blue arrow). Regions of high, satisfactory, and uncertain sensitivity are shown on blue, yellow, and pink backgrounds respectively (see text for details).
The high sensitivity of the DHPLC protocol is underscored by our previous success in demonstrating that the clinically unaffected mother of an ADH family was a mosaic for an activating \textit{CASR} mutation \cite{Hendy et al. 2003}. In that instance, identification of the mutation in 5–10% of the

\begin{table}
\centering
\caption{Positive controls for denaturing high performance liquid chromatography (DHPLC) evaluation}
\begin{tabular}{|l|l|l|l|l|l|}
\hline
\textbf{Numbers} & \textbf{Mutation\textsuperscript{a}} & \textbf{Amplicon} & \textbf{Trivial name} & \textbf{Nucleotide\textsuperscript{b} position} & \textbf{Nucleotide change} & \textbf{References\textsuperscript{c}} \\
\hline
2 & FHH & E2-2 & F42S & 125 & TTT > TCT & Novel \\
3 & FHH & E2-2 & P55L & 164 & CGG > CTG & Heath \textit{et al.} (1996)\textsuperscript{*} \\
5 & FHH & E3-1 & I81M & 243 & ATA > ATG & Novel \\
6 & ADH & E3-2 & N118K & 354 & AAC > AAA & Pearce \textit{et al.} (1996)\textsuperscript{*} \\
7 & ADH & E3-2 & L125F & 373 & CTT > TTT & Novel \\
8 & ADH & E3-2 & C129R & 385 & TGC > CGC & Novel \\
9 & FHH & E3-2 & T138M & 413 & AGG > ATG & D'Souza-Li \textit{et al.} (2002) \\
10 & FHH & E3-2 & G143R & 427 & GGA > AGA & Novel \\
11 & FHH & E3-2 & G158R & 472 & GGG > AGG & Novel \\
13 & FHH & E4-1 & S166G & 496 & AGT > GGT & Novel \\
14 & FHH & E4-1 & R220W & 658 & CCG > TGG & D'Souza-Li \textit{et al.} (2002)\textsuperscript{*} \\
15 & ADH & E4-1 & E228K & 682 & GAG > AAG & Novel \\
17 & FHH & E6 & G549R & 1645 & GGG > AGG & D'Souza-Li \textit{et al.} (2002) \\
18 & FHH & E6 & C562Y & 1685 & TGC > TAC & Burski \textit{et al.} (2002)\textsuperscript{*} \\
19 & FHH & E6 & C563G & 1693 & TGT > GGT & Novel \\
20 & FHH & E7-1 & C582Y & 1745 & TGT > TAT & Pearce \textit{et al.} (1995)\textsuperscript{*} \\
23 & FHH & E7-1; E7-2 & C661Y & 1982 & TGC > TAC & Novel \\
24 & FHH & E7-2 & R680H & 2039 & CGC > CAC & Arunchaiva \textit{et al.} (1998)\textsuperscript{*} \\
26 & FHH & E7-3 & I761del & 2281 & del ATG & Novel \\
28 & ADH & E7-3 & N802I & 2405 & AAT > ATT & Novel \\
29 & ADH & E7-3; E7-4 & G830S & 2488 & GGC > AGC & Novel \\
30 & ADH & E7-3; E7-4 & F832L & 2494 & TTT > CTT & Novel \\
31 & ADH & E7-3; E7-4 & F832S & 2495 & TTT > TCT & Dreiman \textit{et al.} (2001) \\
\hline
\textit{a}FHH, familial hypocalciuric hypercalcemia; ADH, autosomal dominant hypocalcemia; Poly, polymorphism. \\
\textit{b}GenBank: X81086, the A of the initiation codon ATG is nucleotide +1. \\
\textit{c}Mutations are either previously identified by us or others (reference given) or novel. In some cases, the same mutation previously reported by another group has been identified by us in a different family or individual (reference with asterisk).
\end{tabular}
\end{table}

Figure 2 DHPLC chromatograms for wild-type versus \textit{CASR} variants (T263M and I283T) found in fragment E4-2, analyzed at 63.0 °C.
individual’s leukocyte DNA was not achieved by conventional analysis.

Further improvements to the present DHPLC method can be anticipated. As each novel mutation is catalogued, it is assessed as a potential positive control, both for saturation of all melting domains within the DHPLC amplicons, but also for sequencing. Eventually, they may be of unique value in a DHPLC quality assurance consortium of the sort described by Schollen et al. (2005). Also, at present, CASR sequence that includes the two promoters is not being interrogated and mutations in that region affecting gene transcription would be missed. The analysis of our set of positive controls (Table 2) clearly indicate that small deletions/insertions within protein-coding exons are readily detected, but larger insertions/deletions affecting the amplification step may not be so readily ascertained. To detect loss or gain of DNA representing an exon (or more) of the CASR gene, methods such as quantitative multiplex PCR of short fluorescent fragments (QMPSF – Houdayer et al. 2004) or multiplex ligation-dependent probe amplification (MLPA – Sellner & Taylor 2004) should be developed.

Novel and recurrent CASR mutations

The mutations in our positive control panel are either previously published or represent recurrent or novel mutations. Therefore, we are able to report here eight novel and five recurrent FHH mutations, and six novel and two recurrent ADH mutations. With one exception, all are missense. The majority of the new FHH mutations are within the ECD of the CASR (F42S, I81M, G143R, G158R, S166G, and C565G), with C661Y within TM-2 and I761del within extracellular loop-2. The scattering of mutations is consistent with the notion of CASR

Table 3 Bioinformatics analysis of FHH missense mutations

<table>
<thead>
<tr>
<th>Numbers</th>
<th>Trivial name</th>
<th>Conservedb</th>
<th>PHEN scorec</th>
<th>SIFT scored</th>
<th>Functional analysis</th>
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<td>3</td>
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</table>

*Mutation numbers in Table 2.

b+, amino acid conserved in CASR of all species; +*, conserved except in Takifugu rubripes.

cScore interpretation: 0, benign; ++, possibly damaging; ++, probably damaging.

dScore interpretation: 0, tolerated; +, affect function.

*eFunctional analysis: N, No; Y, Yes (impaired function).
having multiple functional components that collectively contribute to activity and that a critical mutation in any one of them can cause major impairment (D’Souza-Li et al. 2002). The new ADH mutations are equally divided within the ECD (L125F, C129R, and E228K) and the intracellular loop-3, TM-7, extracellular loop-3 part of the TMD where ADH mutations are known to cluster (see www.casrdb.mcgill.ca (CASRdb)).

Bioinformatics

Predictive programs are increasingly used as adjuncts in the assessment of whether particular missense variants identified in molecular diagnostic testing are likely to negatively affect protein function. We had the opportunity to classify the 16 FHH mutants in the positive control panel as damaging or benign (PolyPhen), or not tolerated or tolerated (SIFT). While most of the mutations were flagged by both programs as potentially deleterious, a few were not so identified. These included (more so for PolyPhen than SIFT) CASR mutants that have been characterized by in vitro functional analysis as being impaired relative to wild type. While the programs provide insightful information in most cases, such information as relates to clinical genetics and genetic counseling should be used with caution (Tchernitchko et al. 2004).

Summary

In conclusion, use of the DHPLC method for CASR mutation searches is as effective as standard sequencing protocols, judging by the lack of discordance for a blind survey of positive and negative controls. DHPLC provides a rapid means to screen for CASR mutations in hypercalcemic families or individuals, and CASR testing provides a critical contribution to the differential diagnosis of hypercalcemic states.

Declaration of interest

None of the authors have any conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


Dreiman D, Hendy GN, Alon U & Geffner M 2001 Normalization of serum calcium, phosphorus, and magnesium with homeopathic PTH in a child with hypocalcemic hypercalciuria (HCCH) and a mutation of the calcium-sensing receptor gene. Program and Abstracts of the 80th Annual Meeting of the Endocrinology Society P3-125 475.


Ng PC & Henikoff S 2003 SIFT: predicting amino acid changes that affect protein function. Nuclear Receptor Receptor Signaling 31 3812–3814.


