Calcium-sensing receptor mutations and denaturing high performance liquid chromatography

David E C Cole, Francisco H J Yun, Betty Y L Wong, Andrew Y Shuen, Ronald A Booth, Alfredo Scillitani[1], Svetlana Pidasheva[2], Xiang Zhou[2], Lucie Canaff[2] and **Geoffrey N Hendy[2]**

Departments of Laboratory Medicine and Pathobiology, Medicine, and Genetics, University of Toronto, Ontario, Canada M5G 1L5,

[1]Endocrinology Unit, Hospital Casa Sollievo della Sofferenza, Istituto di Ricovero e Cura a Carrattere Scientifico, San Giovanni Rotondo (Foggia), 71013 Italy

[2]Hormones and Cancer Research Unit, Calcium Research Laboratory, Departments of Medicine, Physiology, and Human Genetics, Royal Victoria Hospital, McGill University, Room H4.67, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1

(Correspondence should be addressed to G N Hendy; Email: geoffrey.hendy@mcgill.ca)

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 'calciostat', 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. Hereditable 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 3q13·3-q21 (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 NH_2-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

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handful in the intracellular tail are deletions. Activating mutations are more tightly clustered in a 150-amino acid stretch in the first NH_2-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), *RB1* (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′-gcccccgccg-3′), and a 20-bp sequence (5′-gcggcccgccgcccccgccg-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

	Size (bp)	Primer sequences[a]	PCR annealing temperature	DHPLC initial %B	Elution temperature (°C)
Amplicon					
E2	305	F: 5′-**gcccccgccg**CTCCTAGCTGTCTCATCCCTTG-3′ (5′UTR)[b] R: 5′-GTTTGGTGCAGCTTTCTCC-3′ (intronic)	60	53	60, 63
E3-1	202	F: 5′-**gcgtcccg**GCTTCCCATTTTCTTCCACTT-3′ (intronic) R: 5′-ACCAAACTCAGGGTGGCTTC-3′	60	50	59·5, 60·5
E3-2	264	F: 5′-**gcccccgccg**TTGCAACACCGTTTCTGAGG-3′ R: 5′-**gcgtcccg**GCCTGCTTCTTCTGATCCTG-3′ (intronic)	60	53	60·5, 62, 63
E4-1	398	F: 5′-**gcccccgccg**TCATTCACCATGTTCTTGGTTC-3′ (intronic) R: 5′-**gcgtcccg**CTTGATGAGGGGCTCAAGAT-3′	60	57	60·5, 61·5
E4-2	377	F: 5′-**gcgtcccg**CATGTGGTAGAGGTGATTCAAAA-3′ R: 5′-**gcgtcccg**GAAAGGTGTCCACAGGTAAAGG-3′	60	56	60·5, 61·5, 63
E4-3	367	F: 5′-TTTAACTGCCACCTCCAAGAAG-3′ R: 5′-GCAGCCCAACTCTGCTTTATT-3′ (intronic)	60	56	60, 61
E5	343	F: 5′-CAGGGCACAGCCTACCTAAT-3′ (intronic) R: 5′- CCTGGTGGAGACATCTGGTT-3′ (intronic)	60	55	60
E6	221	F: 5′-**gcgtcccg**CTGGCCCCTGACCCTACAAC-3′ (intronic) R: 5′-**gcgtcccg**ACAGTGCCCAAGAGGGGTTC-3′ (intronic)	60	51	62, 63
E7-1	341	F: 5′-**gcccccgccg**CACTCACACATTTTAGTCTGTGC-3′ (intronic) R: 5′- AAGAACAGGGAGCTGGAGAA-3′	60	55	62
E7-2	284	F: 5′-**gcccgccgcccccgccg**GCTCTCCTACCTCCTCCTCTTC-3′ R: 5′-**gccgccccccgcccg**GAAGGTGCAGAGGAAAACCA-3′	60	54	63·5, 64·5
E7-3	414	F: 5′-CTGGTGTTTGAGGCCAAGAT-3′ R: 5′-GATGGCAATCACCTCTACGG-3′	58	57	62, 63
E7-4	432	F: 5′-**gcgtcccg**CAGCCTATGCCAGCACCTAT-3′ R: 5′-**cggcccgccgcccccgcccg**CTGAGATCGTTGCTGCTGTG-3′	62	57	62, 65
E7-5	455	F: 5′-CTAACCCAGCAAGAGCAGCA-3′ R: 5′-TCTCCCTAGCCCAGTCTTCTC-3′ (3′UTR)	60	NA[c]	NA

[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 Transgenomic WAVE system equipped with a DNASep 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 Na_2EDTA) with buffer B (25% acetonitrile, 0·1 M TEAA, and 0·1 M Na_2EDTA); 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 I283T, 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

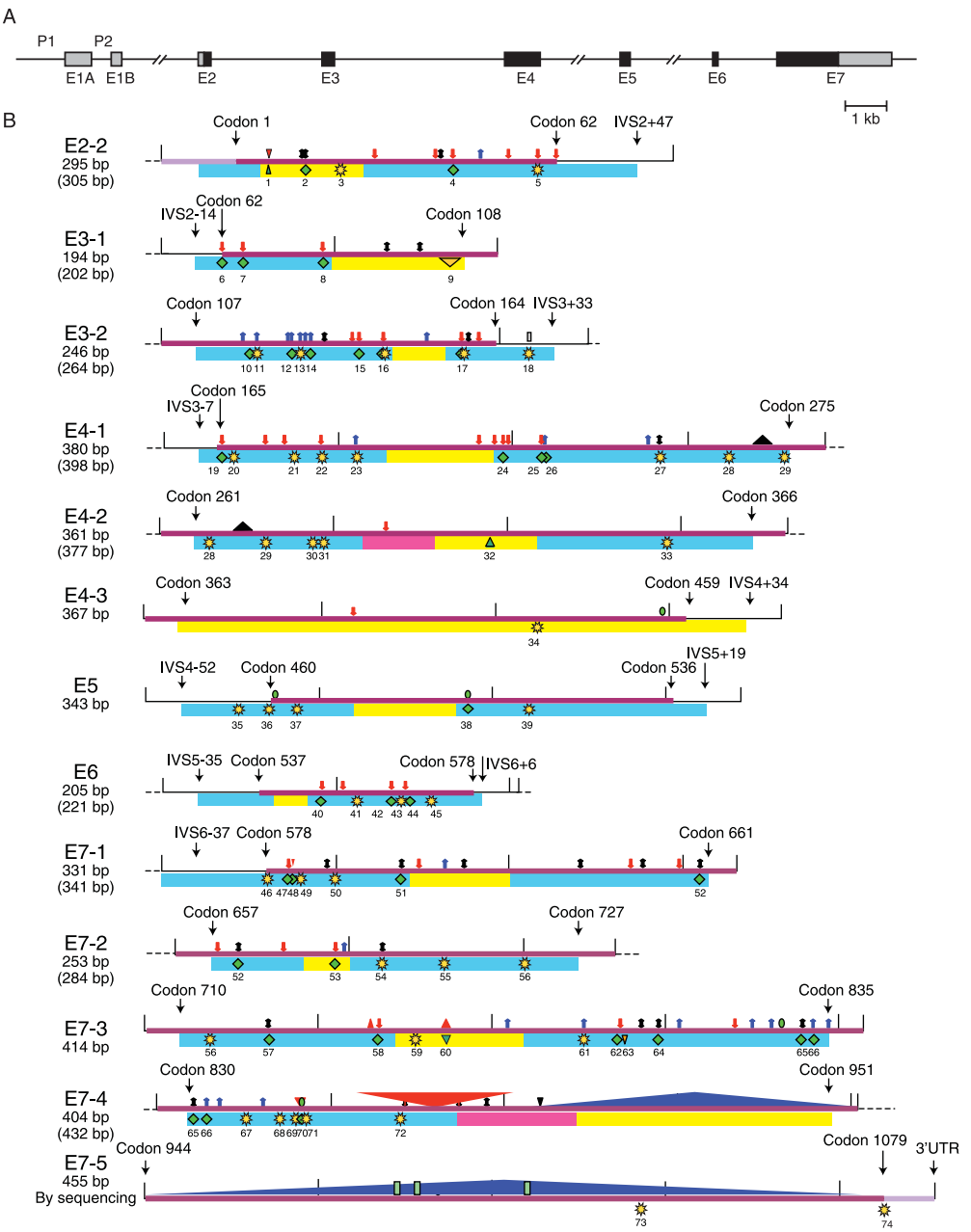

Figure 1 Panel (A) genomic organization of the *CASR* that is comprised of eight exons. Untranslated regions, exons 1A and 1B, part of exon 2, and part of exon 7 are in gray, while part of exon 2, exons 3–6, and part of exon 7 that are protein-coding are in black. P1, promoter 1, P2, promoter 2. Panel (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 (▮), activating (▮), unknown (▮) – silent mutations (●), insertion/deletion (▲,▼), and SNPs (▯). Mutations found through our in-house mutation screening are indicated as missense (✱) or deletion (▽). Positive controls are presented as (◆). 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 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

Table 2 Positive controls for denaturing high performance liquid chromatography (DHPLC) evaluation

	Mutation[a] type	Amplicon	Trivial name	Nucleotide[b] position	Nucleotide change	References[c]
Numbers						
1	FHH	E2-2	C7fs-2X47	19	TGC >TTGC	D'Souza-Li *et al.* (2002)
2	FHH	E2-2	F42S	125	TTT > TCT	Novel
3	FHH	E2-2	P55L	164	CCG > CTG	Heath *et al.* (1996)*
4	FHH	E3-1	R66H	197	CGT > CAT	Pidasheva *et al.* (2006)
5	FHH	E3-1	I81M	243	ATA > ATG	Novel
6	ADH	E3-2	N118K	354	AAC > AAA	Pearce *et al.* (1996)*
7	ADH	E3-2	L125F	373	CTT > TTT	Novel
8	ADH	E3-2	C129R	385	TGC>CGC	Novel
9	FHH	E3-2	T138M	413	ACG > ATG	D'Souza-Li *et al.* (2002)
10	FHH	E3-2	G143R	427	GGA>AGA	Novel
11	FHH	E3-2	G158R	472	GGG > AGG	Novel
12	Poly	E4-1	IVS3+19	492+19	A>G	Yun *et al.* (2007)
13	FHH	E4-1	S166G	496	AGT>GGT	Novel
14	FHH	E4-1	R220W	658	CGG > TGG	D'Souza-Li *et al.* (2002)*
15	ADH	E4-1	E228K	682	GAG > AAG	Novel
16	Poly	E5	S497S	1491	TCC > TCT	Yun *et al.* (2007)
17	FHH	E6	G549R	1645	GGG > AGG	D'Souza-Li *et al.* (2002)
18	FHH	E6	C562Y	1685	TGC > TAC	Burski *et al.* (2002)*
19	FHH	E6	C565G	1693	TGT >GGT	Novel
20	FHH	E7-1	C582Y	1745	TGT > TAT	Pearce *et al.* (1995)*
21	FHH	E7-1	N583X	1747	AAC > TAAC	Pidasheva *et al.* (2006)
22	ADH	E7-1	E604K	1810	GAG > AAG	Alvarez-Hernandez *et al.* (2003)*
23	FHH	E7-1; E7-2	C661Y	1982	TGC > TAC	Novel
24	FHH	E7-2	R680H	2039	CGC > CAC	Arunchaiva *et al.* (1998)*
25	Poly	E7-3	P748P	2244	CCC > CCG	Yun *et al.* (2007)
26	FHH	E7-3	I761del	2281	del ATG	Novel
27	FHH	E7-3	R795W	2383	CGG > TGG	D'Souza-Li *et al.* (2002)
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	Dreimane *et al.* (2001)
32	FHH	E7-4	C851fs-2X981	2551	ins CCAG	D'Souza-Li *et al.* (2002)

[a]FHH, familial hypocalciuric hypercalcemia; ADH, autosomal dominant hypocalcemia; Poly, polymorphism.
[b]GenBank: X81086, the A of the initiation codon ATG is nucleotide +1.
[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).

evaluation of positive and negative controls, previously established by conventional sequencing, yielded a concordance rate of 100%. 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 *CASR* mutation (Hendy *et al.* 2003). In that instance, identification of the mutation in 5–10% of the

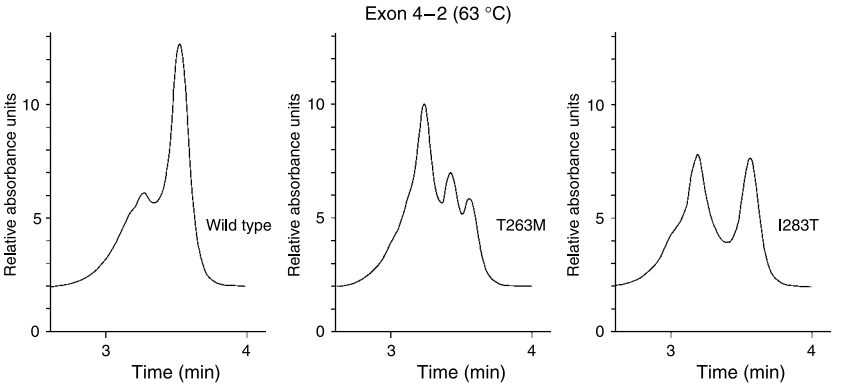

Figure 2 DHPLC chromatograms for wild-type versus *CASR* variants (T263M and I283T) found in fragment E4-2, analyzed at 63.0 °C.

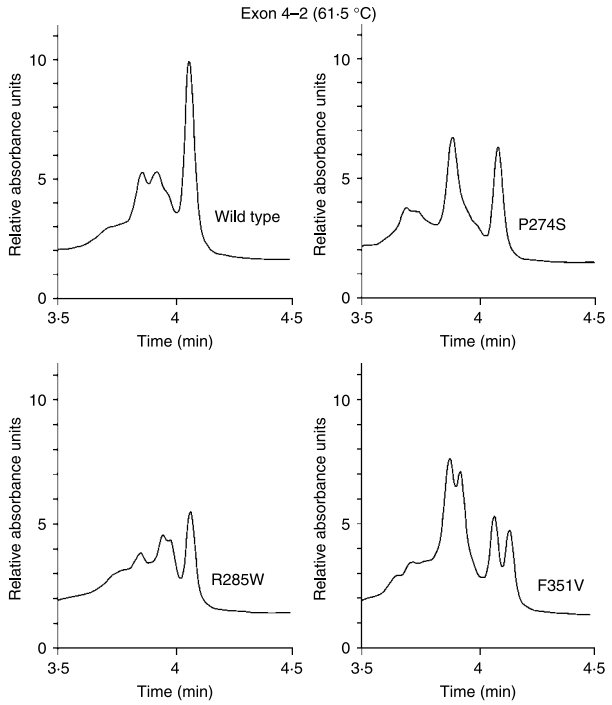

Figure 3 DHPLC chromatograms for wild-type versus *CASR* variants (P274S, R285W, and F351V) found in fragment E4-2, analyzed at 61.5 °C.

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

Numbers[a]	Trivial name	Conserved[b]	PHEN score[c]	SIFT score[d]	Functional[e] analysis	References
2	F42S	+	0	0	N	Novel
3	P55L	+	++	+	Y	Bai *et al.* (1996)
4	R66H	+	0	0	Y	Pidasheva *et al.* (2006)
5	I81M	+	++	+	N	Novel
9	T138M	+	0	+	Y	Bai *et al.* (1996)
10	G143R	+	++	+	N	Novel
11	G158R	+*	+	0	N	Novel
13	S166G	+	0	0	N	Novel
14	R220W	+	++	+	Y	D'Souza-Li *et al.* (2002)
17	G549R	+	0	+	Y	D'Souza-Li *et al.* (2002)
18	C562Y	+	++	+	N	Burski *et al.* (2002)
19	C565G	+	++	+	N	Novel
20	C582Y	+	++	+	N	Pearce *et al.* (1995)
23	C661Y	+	+	+	N	Novel
24	R680H	+	++	+	N	Arunchaiva *et al.* (1998)
27	R795W	+	++	+	Y	Bai *et al.* (1996)

[a]Mutation numbers in Table 2.
[b]+, amino acid conserved in CASR of all species; +*, conserved except in *Takifugu rubripes*.
[c]Score interpretation: 0, benign; +, possibly damaging; ++, probably damaging.
[d]Score interpretation: 0, tolerated; +, affect function.
[e]Functional 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

Alvarez-Hernandez D, Santamaria I, Rodriguez-Garcia M, Iglesias P, Delgado-Lillo R & Cannata-Andia JB 2003 A novel mutation in the calcium-sensing receptor responsible for autosomal dominant hypocalcemia in a family with two uncommon parathyroid hormone polymorphisms. *Journal of Molecular Endocrinology* **31** 255–262.

Arunchaiva S, Pollak MR, Seidman CA, Pinhas-Hamiel O, Welch TR, Tsang RC & Smith EP 1998 Marked hypercalcemia in a five-month-old male associated with heterozygous point mutation in the calcium-sensing receptor gene. *Program and Abstracts of the 80th Annual Meeting of the Endocrinology Society* **P3-535** 495.

Bai M, Quinn SJ, Trivedi S, Kifor O, Pearce SHS, Pollak MR, Krapcho KJ, Hebert SC & Brown EM 1996 Expression and characterization of inactivating and activating mutations of the human Ca^{2+}o-sensing receptor. *Journal of Biological Chemistry* **271** 19537–19545.

Brown EM 2007 Clinical lessons from the calcium-sensing receptor. *Nature Clinical Practice. Endocrinology & Metabolism* **3** 122–133.

Burski K, Torjussen B, Paulsen AQ & Bollerslev J 2002 Parathyroid adenona in a subject with familial hypocalciuric hypercalcemia: coincidence or causality? *Journal of Clinical Endocrinology and Metabolism* **87** 1015–1016.

Canaff L & Hendy GN 2002 Human calcium-sensing receptor gene: vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. *Journal of Biological Chemistry* **277** 30337–30350.

Chikatsu N, Fukumoto S, Takeuchi Y, Suzawa M, Obara T, Matsumoto T & Fujita T 2000 Cloning and characterization of two promoters for the human calcium-sensing receptor (CaSR) and changes of CaSR expression in parathyroid adenomas. *Journal of Biological Chemistry* **275** 7553–7557.

Cole DEC, Peltekova VD, Rubin LA, Hawker GA, Vieth R, Liew CC, Hwang DM, Evrovski J & Hendy GN 1999 A986S, polymorphism of the calcium-sensing receptor and circulating calcium concentrations. *Lancet* **353** 112–115.

Cole DEC, Vieth R, Trang HM, Wong BY-L, Hendy GN & Rubin LA 2001 Association between total serum calcium and the A986S polymorphism of the calcium-sensing receptor gene. *Molecular Genetics and Metabolism* **72** 168–174.

Crépin M, Pigny P, Escande F, Bauters CC, Calender A, Lefevre S, Buisine M-P, Porchet N & Odou M-F 2006 Evaluation of denaturing high performance liquid chromatography for the mutational analysis of the MEN1 gene. *Journal of Molecular Endocrinology* **36** 369–376.

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

D'Souza-Li L, Yang B, Canaff L, Bai M, Hanley DA, Bastepe M, Salisbury SR, Brown EM, Cole DEC & Hendy GN 2002 Identification and functional characterization of novel calcium-sensing receptor mutations in familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia. *Journal of Clinical Endocrinology and Metabolism* **87** 1309–1318.

Garrett JE, Capuano IV, Hammerland LG, Hung BC, Brown EM, Hebert SC, Nemeth EF & Fuller F 1995 Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *Journal of Biological Chemistry* **270** 12919–12925.

Gerhardus A, Schleberger H, Schlegelberger B & Gadzicki D 2007 Diagnostic accuracy of methods for the detection of BRCA1 and BRCA2 mutations: a systematic review. *European Journal of Human Genetics* **15** 619–627.

Guarnieri V, Scillitani A, Battista C, Muscarella LA, Coco M, D'Agruma L, Sacco M, Canaff L, Yun F, Wong BYL, Hendy GN & Cole DEC 2008 Molecular diagnostic strategies for mutations of the calcium-sensing receptor gene: application to a large cohort of patients with different hypercalcemic syndromes. *Journal of Bone and Mineral Research* **23** 423.

He N, Zahirieh A, Mei Y, Lee B, Senthilnathan S, Wong B, Mucha B, Hildebrandt F, Cole DEC, Cattran D *et al.* 2007 Recessive NPHS2 (podocin) mutations are rare in adult-onset idiopathic focal segmental glomerulosclerosis. *Clinical Journal of the American Society of Nephrology* **2** 31–37.

Heath H III, Odelberg S, Jackson CE, Teh BT, Hayward N, Larsson C, Buist NR, Krapcho KJ, Hung BC, Capuano IV *et al.* 1996 Clustered inactivating mutations and benign polymorphisms of the calcium receptor gene in familial benign hypocalciuric hypercalcemia suggest receptor functional domains. *Journal of Clinical Endocrinology and Metabolism* **81** 1312–1317.

Hendy GN & Cole DEC 2007 Parathyroid Disorders. In *Emery and Rimoin's Principles and Practice of Medical Genetics* 5th edition, vol 2 ch 89, pp 1951–1979. Eds DL Rimoin, JM Connor, RE Pyeritz & BE Korf. Edinburgh: Churchill Livingstone.

Hendy GN, D'Souza-Li L, Yang B, Canaff L & Cole DEC 2000 Mutation update, mutations in the calcium-sensing receptor (CASR) in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism and autosomal dominant hypocalcemia. *Human Mutation* **16** 281–296.

Hendy GN, Minutti C, Canaff L, Pidasheva S, Yang B, Nouhi Z, Zimmerman D, Wei C & Cole DEC 2003 Recurrent familial hypocalcemia due to germline mosaicism for an activating mutation of the calcium-sensing receptor gene. *Journal of Clinical Endocrinology and Metabolism* **88** 3674–3681.

Houdayer C, Gauthier-Villars M, Lauge A, Pagès-Berhouet S, Dehainault C, Caux-Moncoutier V, Karczynski P, Tosi M, Doz E, Desjardins L *et al.* 2004 Comprehensive screening for constitutional RB1 mutations by DHPLC and QMPSF. *Human Mutation* **23** 193–202.

Janicic N, Soliman E, Pausova Z, Seldin MF, Rivière M, Szpirer J, Szpirer C & Hendy GN 1995 Mapping of the Ca^{2+}-sensing receptor gene to human chromosome 3q13.3-21 by fluorescence *in situ* hybridization, and localization to rat chromosome 11 and mouse chromosome 16. *Mammalian Genome* **6** 798–801.

Law WM & Heath H III 1985 Familial benign hypercalcemia (hypocalciuric hypercalcemia): clinical and pathogenetic study of 21 families. *Annals of Internal Medicine* **102** 511–519.

Lenato GM, Lastella P, Di Giacomo MC, Resta N, Suppressa P, Pasculli G, Sabbà C & Guanti G 2006 DHPLC-based mutations analysis of ENG and ALK-1 genes in HHT Italian populations. *Human Mutation* **27** 213–214.

Marx SJ, Attie MF, Levine MA, Spiegel AM, Downs RWJ & Lasker RD 1981 The hypocalciuric or benign variant of familial hypercalcemia: clinical and biochemical features of fifteen families. *Medicine* **60** 397–412.

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

Oefner PJ & Underhill PA 1998 DNA mutation detection using denaturing high-performance liquid chromatography (DHPLC). In *Current Protocols in Human Genetics*, vol 19:7.10.1-7.10.12, Eds NC Dracopoli, JL Haines, BR Korf, DT Moir, CC Morton, CE Seidman, JG Seidman & DR Smith. New York: Wiley & Sons.

Pearce SHS, Trump D, Wooding C, Besser GM, Chew S, Heath D, Hughes I & Thakker RV 1995 Calcium-sensing receptor mutations in familial benign hypercalcemia and neonatal hyperparathyroidism. *The Journal of Clinical Investigation* **96** 2683–2692.

Pearce SHS, Williamson C, Kifor O, Bai M, Coulthard MG, Davies M, Lewis-Barned N, McCredie D, Powell H, Kendall-Taylor P *et al.* 1996 A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *New England Journal of Medicine* **335** 1115–1122.

Pidasheva S, D'Souza-Li L, Canaff L, Cole DEC & Hendy GN 2004 CASRdb, calcium-sensing receptor locus-specific database for mutations causing familial (benign) hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism and autosomal dominant hypocalcemia. *Human Mutation* **24** 107–111.

Pidasheva S, Grant M, Canaff L, Ercan O, Kumar U & Hendy GN 2006 The calcium-sensing receptor (CASR) dimerizes in the endoplasmic reticulum: biochemical and biophysical characterization of novel CASR mutations causing familial hypocalciuric hypercalcemia. *Human Molecular Genetics* **15** 2200–2209.

Pollak MR, Brown EM, Chou Y-HW, Hebert SC, Marx SJ, Steinmann B, Levi T, Seidman CE & Seidman JG 1993 Mutations in the human Ca^{2+}-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* **75** 1297–1303.

Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, Hebert SC, Seidman CE & Seidman JG 1994 Autosomal dominant hypocalcemia caused by a Ca^{2+}-sensing receptor gene mutation. *Nature Genetics* **8** 303–307.

Ramensky V, Bork P & Sunyaev S 2002 Human non-synonymous SNPs: server and survey. *Nuclear Receptor Signaling* **30** 3894–3900.

Schollen E, Dequeker E, McQuaid S, Vankeirsbilck B, Michils G, Harvey J, van den Akker E, van Schooten R, Clark Z, Schrooten S *et al.* 2005 DDQA, Collaborative Group Diagnostic DHPLC Quality Assurance (DDQA): a collaborative approach to the generation of validated and standardized methods for DHPLC-based mutation screening in clinical genetics laboratories. *Human Mutation* **25** 583–592.

Scillitani A, Guarnieri V, De Geronimo S, Muscarella LA, Battista C, D'Agruma L, Bertoldo F, Florio C, Minisola S, Hendy GN *et al.* 2004 Blood ionized calcium is associated with clustered polymorphisms in the carboxy-terminal tail of the calcium-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* **89** 5634–5638.

Sellner LN & Taylor GR 2004 MLPA and MAPH: new techniques for detection of gene deletions. *Human Mutation* **23** 413–419.

Sunyaev S, Ramensky V, Koch I, Lathe W III, Kondrashov AS & Bork P 2001 Prediction of deleterious human alleles. *Human Molecular Genetics* **10** 591–597.

Tchernitchko D, Goossens M & Wajcmen H 2004 Insilico prediction of the deleterious effect of a mutation: proceed with caution in clinical genetics. *Clinical Chemistry* **50** 1974–1978.

Waller S, Kurzawinski T, Spitz L, Thakker R, Cranston T, Pearce S, Cheetham T & van't Hoff WG 2004 Neonatal severe hyperparathyroidism: genotype/phenotype correlation and the use of pamidronate as rescue therapy. *European Journal of Pediatrics* **163** 589–594.

Xiao W & Oefner PJ 2001 Denaturing high-performance liquid chromatography: A review. *Human Mutation* **17** 439–474.

Yun FHJ, Wong BYL, Chase M, Shuen AY, Canaff L, Thongthai K, Siminovitch K, Hendy GN & Cole DEC 2007 Genetic variation at the calcium-sensing receptor (CASR) locus: Implications for clinical molecular diagnostics. *Clinical Biochemistry* **40** 551–561.

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