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Adaptor protein-2 sigma subunit mutations causing familial hypocalciuric hypercalcaemia type 3 (FHH3) demonstrate genotype–phenotype correlations, codon bias and dominant-negative effects

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Abstract

The adaptor protein-2 sigma subunit (AP2σ) is pivotal for clathrin-mediated endocytosis of plasma membrane constituents such as the calcium-sensing receptor (CaSR). Mutations of the AP2σ Arg15 residue result in familial hypocalciuric hypercalcaemia type 3 (FHH3), a disorder of extracellular calcium (Ca\textsuperscript{2+}) homeostasis. To elucidate the role of AP2σ in Ca\textsuperscript{2+} regulation, we investigated 65 FHH probands, without other FHH-associated mutations, for AP2σ mutations, characterized their functional consequences and investigated the genetic mechanisms leading to FHH3. AP2σ mutations were identified in 17 probands, comprising 5 Arg15Cys, 4 Arg15His and 8 Arg15Leu mutations. A genotype-phenotype correlation was observed with the Arg15Leu mutation leading to marked hypercalcaemia. FHH3 probands harboured additional phenotypes such as cognitive dysfunction. All three FHH3-causing AP2σ mutations impaired CaSR signal transduction in a dominant-negative manner. Mutational bias was observed at the AP2σ Arg15 residue as other predicted missense substitutions (Arg15Gly, Arg15Pro and Arg15Ser), which also caused CaSR loss-of-function, were not detected in FHH probands, and these mutations were found to reduce the numbers of CaSR-expressing cells. FHH3 probands had significantly greater serum calcium (sCa) and magnesium (sMg) concentrations with reduced urinary calcium to creatinine clearance ratios (CCCR) in comparison with FHH1 probands with CaSR mutations, and a calculated index of sCa × sMg/100 × CCCR, which was ≥ 5.0, had a diagnostic sensitivity and specificity of 83 and 86%, respectively, for FHH3. Thus, our studies demonstrate AP2σ mutations to result in a more severe FHH phenotype with genotype-phenotype correlations, and a dominant-negative mechanism of action with mutational bias at the Arg15 residue.

Introduction

Familial hypocalciuric hypercalcaemia (FHH) is an autosomal dominant disorder of extracellular calcium (Ca\textsuperscript{2+}) homeostasis characterized by lifelong mild-to-moderate elevations of serum calcium concentrations, mild hypermagnesaemia, normal or elevated circulating parathyroid hormone (PTH) concentrations and inappropriately low urinary calcium excretion [mean urinary calcium < 50 mg/24 h]. To date, 19 FHH patients and families have been reported in 19 FHH patients and families to date, and these all comprise heterozygous missense substitutions of the AP2σ subunit (AP2σ) Arg15 residue (Arg15Cys, Arg15His and Arg15Leu) (11,12,14). This Arg residue is located in a positively charged region of AP2σ that binds to specific peptide motifs on membrane cargo proteins (18). It is predicted that FHH3-causing Arg15 mutations disrupt binding of the AP2 complex to the intracellular carboxyl terminus of the CaSR, thereby impairing endocytosis of this GPCR (11). This hypothesis is supported by in vitro expression studies that have demonstrated AP2σ mutations to affect CaSR cell-surface expression and signal transduction (11). These studies of AP2σ mutations have highlighted a role for the AP2 endocytic complex in Ca\textsuperscript{2+} homeostasis. To date, 19 FHH3 patients have been reported with AP2σ mutations, comprising 8 Arg15Cys, 4 Arg15His and 7 Arg15Leu. To further elucidate the role and spectrum of AP2σ mutations in the aetiology of the phenotypic features of FHH3, we conducted studies to characterise the structural-functional consequences of AP2σ mutations together with their underlying genetic mechanisms in additional FHH patients, who did not have CaSR or GNA11 mutations. Our study identified AP2σ mutations in 17 hypercalcaemic probands and their families, and further analysis of these AP2σ mutations has revealed the presence of a genotype-phenotype correlation, a mutational bias of the AP2σ Arg15 residue with a...
likely dominant-negative action and a clinical approach to differentiate patients with FHH3 from those with FHH1.

**Results**

**AP2S1 mutations and clinical phenotypes**

DNA sequence analysis of the entire AP2S1 429-bp coding region and 8 exon-intron boundaries was undertaken in 65 unrelated FHH probands without CASR or GNA11 mutations (22 males and 43 females). This revealed the presence of AP2S1 mutations in 17 probands (7 males and 10 females), thereby representing a >25% AP2S1 mutation detection rate in this cohort of FHH patients without CASR and GNA11 mutations. The FHH3-associated mutations only affected the AP2σ2 Arg15 (R15) residue and consisted of five Arg15Cys (R15C), four Arg15His (R15H) and eight Arg15Leu (R15L) mutations (Table 1), all of which had previously been reported to represent pathogenic mutations (11). Two unrelated FHH3 female probands, aged 7 and 15 years, harboured Arg15Leu mutations that were demonstrated to be absent in both of their parents, and hence likely to be arising de novo (Fig. 1). Four FHH3 subjects had symptoms attributable to hypercalcaemia, including lethargy, constipation, widespread musculoskeletal pain and polydipsia (Table 1). Eleven FHH3 probands had clinical features in addition to hypercalcaemia and hypocalciuria (Table 1). In particular, bone mineral density (BMD) was noted to be low (T-score < −1.0 or Z-score < −2.0) at the lumbar spine or femoral neck in 5 of 10 patients aged 14 to 64 years (Table 1). The low BMD in all five patients was not associated with renal dysfunction, hyperparathyroidism, vitamin D deficiency or thyrotoxicosis. Furthermore, seven FHH3 patients (aged 3–37 years) were noted to have learning disabilities characterized by cognitive deficits and/or behavioural disturbances (Table 1). In addition, the father of a proband with learning disabilities (06/13a, Table 1) also had a cognitive deficit in association with hypercalcaemia. Two of the Arg15Leu probands with cognitive deficits (02/03 and 02/11, Table 1) had short stature with height at or below the third centile, and one of these probands (02/03, Table 1) was found to harbour an atrial septal defect, whereas the other individual (02/11, Table 1) suffered from recurrent episodes of pancreatitis. The pancreatitis in this patient was not due to gallstones or alcohol abuse, and analysis of genes known to be associated with pancreatitis, such as SPINK1, CFTR, PRSS1 and CTRC (19), did not reveal any mutations.

**Genotype–phenotype correlations at the AP2σ2 Arg15 residue**

We examined for phenotypic differences between patients with Arg15Cys, Arg15His or Arg15Leu AP2S1 mutations. Analysis of biochemical and clinical data available for a total of 27 FHH3 probands [17 from this study and 10 from our previously reported study (11)] revealed that patients with the Arg15Leu mutation had significantly greater elevations of serum albumin-adjusted-calcium concentrations when compared with those with the Arg15Cys and Arg15His AP2σ2 mutations (3.06 ± 0.04 mmol/l for Arg15Leu versus 2.83 ± 0.03 mmol/l for Arg15Cys and 2.74 ± 0.03 mmol/l for Arg15His, P < 0.01). Moreover, patients with the Arg15Cys mutation had a significantly greater hypercalcaemia (P < 0.05) than patients harbouring the Arg15His mutation. Such differences were not observed for other biochemical indices of mineral metabolism (Fig. 2).

### Table 1. Clinical and biochemical findings in 17 FHH probands with AP2S1 mutations

| Mutation | Patient | Sex | Family history | Age at presentation/diagnosis (years) | Associated clinical features | Serum Ca<sup>a,b</sup> | PF<sup>+</sup> | Mg<sup>d</sup> | ALP<sup>e,f</sup> | PTH<sup>h</sup> | Urine CCCR<sup>i</sup> | BMD | LS | FN
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<tr>
<td>Arg15Cys</td>
<td>19/12a</td>
<td>M</td>
<td>Yes</td>
<td>48</td>
<td>Nil</td>
<td>2.94 ± 0.23</td>
<td>0.82</td>
<td>110 ± 64.0</td>
<td>45.0 ± 0.009</td>
<td>−1.0 ± 0.2 ± 2.9 ±</td>
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<tr>
<td>03/13a</td>
<td>M</td>
<td>Yes</td>
<td>22</td>
<td>H</td>
<td>2.96 ± 0.08</td>
<td>−98 ± 5.0</td>
<td>41.0 ± 0.004</td>
<td>−</td>
<td>−</td>
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<tr>
<td>16/13</td>
<td>M</td>
<td>Yes</td>
<td>37</td>
<td>H, L</td>
<td>2.80 ± 0.71</td>
<td>68 ± 5.0</td>
<td>50.0 ± 0.003</td>
<td>−1.6 ± 0.2 ±</td>
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<tr>
<td>07/14</td>
<td>F</td>
<td>Yes</td>
<td>16</td>
<td>B</td>
<td>1.50 ± 0.22</td>
<td>115 ± 44.0</td>
<td>44.0 ± 0.004</td>
<td>−1.2 ± 1.4 ±</td>
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<tr>
<td>11/14</td>
<td>M</td>
<td>−</td>
<td>3</td>
<td>L</td>
<td>2.90 ± 1.09</td>
<td>91 ± 368</td>
<td>60.0 ± 0.004</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Arg15His</td>
<td>08/11</td>
<td>F</td>
<td>Yes</td>
<td>33</td>
<td>H</td>
<td>2.76 ± 0.85</td>
<td>74 ± 7.4</td>
<td>0.010 ± 0.2 ± 1.7 ±</td>
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<tr>
<td>03/12</td>
<td>F</td>
<td>Yes</td>
<td>44</td>
<td>Nil</td>
<td>2.80 ± 0.08</td>
<td>65 ± 5.2</td>
<td>0.009 ± 0.0 ± 0.4 ±</td>
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<tr>
<td>19/12b</td>
<td>F</td>
<td>−</td>
<td>19</td>
<td>−</td>
<td>2.74 ± 0.65</td>
<td>104 ± 4.6</td>
<td>0.005 ± 0.0 ±</td>
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<tr>
<td>03/13b</td>
<td>M</td>
<td>Yes</td>
<td>64</td>
<td>Nil</td>
<td>2.72 ± 0.04</td>
<td>103 ± 6.0</td>
<td>−0.6 ± 1.1 ± 0.0 ±</td>
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<td>Arg15Leu</td>
<td>02/03</td>
<td>F</td>
<td>No</td>
<td>15</td>
<td>A, L, S</td>
<td>2.90 ± 0.86</td>
<td>103 ± 4.7</td>
<td>0.015 ± 4.6 ±</td>
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<tr>
<td>13/08</td>
<td>F</td>
<td>No</td>
<td>&lt;1&lt;sup&gt;l&lt;/sup&gt;</td>
<td>L</td>
<td>3.01 ± 0.99</td>
<td>242 ± 15.0</td>
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<tr>
<td>02/11</td>
<td>F</td>
<td>−</td>
<td>14</td>
<td>L, P, S</td>
<td>3.20 ± 1.2</td>
<td>108 ± 31.0</td>
<td>0.005 ± 3.0 ± 2.5 ±</td>
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<tr>
<td>04/11</td>
<td>M</td>
<td>−</td>
<td>11</td>
<td>L</td>
<td>3.00 ± 0.60</td>
<td>314 ± 50.0</td>
<td>0.004 ± 1.4 ± 1.4 ±</td>
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<tr>
<td>09/12</td>
<td>F</td>
<td>−</td>
<td>9</td>
<td>−</td>
<td>2.81 ± 1.0</td>
<td>97 ± 298</td>
<td>96 ± 0.002</td>
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<tr>
<td>06/13a</td>
<td>M</td>
<td>Yes</td>
<td>9</td>
<td>L</td>
<td>3.10 ± 0.78</td>
<td>249 ± 38 ±</td>
<td>0.27 ± 0.0 ±</td>
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<tr>
<td>06/13b</td>
<td>F</td>
<td>No</td>
<td>7</td>
<td>Nil</td>
<td>3.03 ± 0.87</td>
<td>285 ± 40.5</td>
<td>0.001 ± 1.3 ± 0.0 ±</td>
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<tr>
<td>02/14</td>
<td>F</td>
<td>No</td>
<td>26</td>
<td>H</td>
<td>2.95 ± 1.17</td>
<td>98 ± 4.0</td>
<td>0.008 ± 0.0 ±</td>
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Normal serum ranges (6): albumin-adjusted-calcium, 2.10–2.60 mmol/l; ionized calcium, 1.19–1.35 mmol/l; phosphate (Pi), 0.70–1.40 mmol/l; magnesium (Mg), 0.70–1.0 mmol/l; total alkaline phosphatase (ALP) activity, 90–130 U/l; 70–330 U/l; PTH, 70–65 ng/l; 1.3–7.6 pmol/l. Normal urine ranges: calcium-to-creatinine clearance ratio (CCCR), >0.02; calcium-to-creatinine ratio, 0.3–0.7; BMD Z-scores are provided for subjects of <50 years old and BMD T-scores are provided for subjects of ≥50 years old; forearm BMD T-score = −2.5 for proband 03/13b; whole-body BMD Z-score = −2.0 for proband 06/13b; diagnosed in early infancy; A, atrial septal defect; B, irritable bowel syndrome co-segregating with hypercalcaemia in family 07/14; FN, femoral neck; H, hypercalcaemic symptoms; L, mild-to-moderate learning disability; LS, lumbar spine; N, normal; S, short stature (height < third centile); P, pancreatitis; −, not known.
which provides a diagnostic sensitivity of 83% [95% confidence interval (CI) = 61–95] and specificity of 86% (95% CI = 57–98), and positive and negative predictive values of 90% (95% CI = 70–98) and 80% (95% CI = 52–95), respectively.

Phenotypic differences between FHH3 and FHH1
To determine whether FHH3 is associated with any differences in the biochemical phenotype when compared with FHH1, we analysed the serum and urine biochemistry from 51 FHH3 subjects that included affected members of the previously reported multi-generational FHH3 kindreds from Oklahoma (FHHOK) and Northern Ireland (FHHNI) (20–23), and 43 previously reported FHH1 probands, who all harboured CASR mutations (8). This revealed FHH3 patients to have a greater degree of hypercalcaemia than the FHH1 patients (serum adjusted-calcium = 2.87 ± 0.02 mmol/l for FHH3 versus 2.76 ± 0.02 mmol/l for FHH1, P < 0.001) (Fig. 3). FHH3 patients when compared with FHH1 patients also had significantly marked hypermagnesaemia (serum magnesium = 1.04 ± 0.02 mmol/l for FHH3 versus 0.95 ± 0.02 mmol/l for FHH1, P < 0.01) and hypocalciuria (CCCR = 0.004 ± 0.001 for FHH3 versus 0.007 ± 0.001 for FHH1, P < 0.01) (Fig. 3). There were no significant differences in the serum concentrations of phosphate, alkaline phosphatase (ALP) activity or PTH between the FHH3 and FHH1 patients (Fig. 3). As serum adjusted-calcium, serum magnesium and CCCR values were significantly different between FHH3 and FHH1 patients, we investigated whether the combined use of these biochemical parameters could be utilized to discriminate between these two hypercalcaemic disorders. Based on the observation that serum adjusted-calcium (sCa) and serum magnesium (sMg) values are elevated, whereas CCCR is reduced in FHH3, a calculated index (sCa × sMg/100 × CCCR), designated CMCR, was determined for each of the subjects in the FHH1 and FHH3 groups (Fig. 3) with all biochemical parameters being measured in millimole per litre. Receiver-operator curve (ROC) analysis of the CMCR index (Fig. 3) revealed an area-under-the-curve (AUC) of 0.85 (P < 0.001) and an optimal cut-off value of 5.0 for discriminating between FHH3 and FHH1, which provides a diagnostic sensitivity of 83% [95% confidence

Analysis of AP2e2 codon 15 mutation bias
The present study has identified 17 hypercalcaemic patients with AP2S1 mutations that all affected the AP2e2 Arg15 residue, and previous studies have reported 19 AP2S1 mutations that all affect the Arg15 residue (11,12,14) yielding a total of 36 FHH3-causing mutations identified to date. These 36 AP2S1 mutations involving substitutions of Arg15 comprise 13 Arg15Cys, 8 Arg15His and 15 Arg15Leu. Analysis of the DNA sequence of the Arg15 codon reveals it to be CGC indicating that missense substitutions affecting the first or second nucleotides would be predicted to be non-synonymous and lead to one of six possible amino acid substitutions, which comprise Cys, Gly, His, Leu, Pro and Ser (Fig. 4). This expected observation of six different amino acid substitutions contrasts significantly (P < 0.0001, Chi-squared test) with the three Arg15-mutant substitutions observed in FHH3 patients.

To investigate the potential occurrence of the Arg15Cys, Arg15His and Arg15Leu, and the absence of Arg15Gly, Arg15Pro and Arg15Ser variants, we assessed the effects of all six potential Arg15 mutants on the structure and function of AP2. Analysis of the AP2 complex crystal structure predicted that all the six Arg15 missense substitutions, including the Arg15Gly, Arg15Pro and Arg15Ser variants that have not been observed in FHH3, would impair AP2 complex function by disrupting a key polar contact between the AP2 complex and the cargo protein dileucine recognition motif (Fig. 4). Thus, all the six Arg15 missense substitutions would significantly alter the structure, and we therefore determined their functional consequences on CaSR activity, by expressing them in HEK293 cells that stably expressed the CaSR
Transient transfection of the wild-type or mutant AP2S1-pBI-CMV4-RFP expression constructs, or vector containing the red fluorescence protein (RFP) reporter gene alone, was undertaken in the HEK-CaSR cells (9,11). Expression of the CaSR and RFP, which represents a surrogate of AP2σ2 expression, was detected by immuno-fluorescence and western blotting of whole-cell lysates (Fig. 5), and the responses of Ca2+ concentrations to alterations in Ca2+o concentrations were then assayed by flow cytometry (9,11).

HEK-CaSR cells expressing each of the six Arg15 missense AP2σ2 variants (i.e. the three observed Arg15Cys, Arg15His and Arg15Leu and the three non-observed Arg15Gly, Arg15Pro and Arg15Ser AP2σ2 variants) were found to have half-maximal effective concentration (EC50) values that were significantly higher (P < 0.0001) than cells expressing the wild-type AP2σ2 protein. Thus, the three non-observed Arg15 AP2σ2 variants decreased the sensitivity of HEK-CaSR cells to Ca2+o concentrations in a similar manner to the three FHH3-causing AP2σ2 mutations (Fig. 5 and Table 2). However, cells expressing the non-observed Arg15Gly, Arg15Pro or Arg15Ser AP2σ2 mutants were found to have significantly reduced increases in cell numbers over a 24-h period, when compared with cells expressing wild-type or FHH3-mutant AP2σ2 proteins (Fig. 5). These results indicate that the Arg15Gly, Arg15Pro and Arg15Ser AP2σ2 mutants are likely not observed as they are associated with an impairment of cell growth, when compared with wild-type or the FHH3-associated mutant AP2σ2 proteins (Fig. 5 and Supplementary Material, Fig. S1).
Dominant-negative effects of AP2S1 mutations

FHH3 is associated with heterozygous loss-of-function AP2σ mutations, and these could be causing the disease by either haploinsufficiency (i.e. a reduced dosage of the wild-type AP2σ protein) or dominant-negative effects on the heterotetrameric AP2 complex. To investigate these genetic mechanisms, we studied the effects of altering the dosage of wild-type and mutant...
AP2σ proteins on the EC_{50} responses of HEK-CaSR cells that were transiently transfected with wild-type or mutant AP2S1-pBl-CMV4-RFP expression constructs. We used the bidirectional AP2S1-pBl-CMV4-RFP vector as it expresses RFP and AP2σ at equivalent levels, thereby enabling RFP expression to be used as a surrogate for AP2σ expression (9). We selected populations of

**Figure 4.** Observed and predicted mutations at the AP2S1 Arg15 (R15) residue. (A) Schematic representation of possible nucleotide and amino acid substitutions affecting codon 15. Substitutions affecting the first or second nucleotide of codon 15 (CGC) are predicted to lead to one of six possible non-synonymous mutations or variants, which are Cys15 (TGC), Gly15 (GGC), His15 (CAC), Leu15 (CTC), Pro15 (CCC) or Ser15 (AGC). Substitutions affecting the third nucleotide of the CGC triplet are predicted to lead to synonymous variants only. (B) Crystal structure of the α-subunit (green) and σ2-subunit (blue) of the AP2 heterotetrameric complex bound to an acidic (Gln-containing) dileucine cargo protein motif [PDB file 2JKR (18)]. The key polar contacts (black dashed lines) between the σ2-subunit Arg15 residue (σ2Arg15, red) and α-subunit Arg21 residue (αArg21, grey), and a Gln residue located four residues from the first Leu residue of the acidic dileucine motif [Gln(Leu-4), orange], are shown. (C–Q) Structural analysis of six potential Arg15 mutations demonstrating that the observed mutants (Cys15, His15 and Leu15) and non-observed mutants (Gly15, Pro15 and Ser15) are all predicted to result in the loss of the key polar contact with the cargo protein Gln(Leu-4) residue.
cells with increasing levels of RFP expression by flow cytometry (Fig. 6) and assessed the effects of altering wild-type and mutant AP2σ2 dosage by measuring the Ca²⁺ responses of the HEK-CaSR cells to changes in Ca²⁺o concentrations, and determining the linear regression of mean EC₅₀ values against expression levels of wild-type and mutant AP2σ2 proteins. In this experiment, loss-of-function mutations with dominant-negative actions, which would exert greater effects with increasing protein concentrations, will show a positive correlation between mutant protein concentration and EC₅₀. However, loss-of-function mutations associated with haploinsufficiency would not exert effects on the wild-type protein, whose concentration would remain constant, and hence there would be absent correlation between mutant protein concentrations and EC₅₀ values (Fig. 6).

The results of such linear regression analyses revealed that increased expression levels of FHH3-causing Arg15-mutant AP2σ2 proteins progressively impaired the sensitivity of HEK-CaSR cells, as highlighted by a significantly positive relationship
Figure 6. Comparison of EC50 values for HEK-CaSR cells transfected with increasing amounts of wild-type (WT) or mutant AP2S1-pBI-CMV4-RFP expression constructs.

(A) During flow cytometry, fluorescence over a 1–10 000 range can be detected. The first large peak represents cells not expressing RFP but displaying low levels of auto-fluorescence. Cells displaying increased fluorescence with respect to this population reflect those expressing RFP. Cells from five different RFP fluoroscences were selected on the basis of mean fluorescence (gate 1 log mean fluorescence = 50–100, gate 2 log mean fluorescence = 125–250, gate 3 log mean fluorescence = 500–1000, gate 4 log mean fluorescence = 1250–2500 and gate 5 log mean fluorescence = 5000–10000), consistent with a fluorescence ranging from between 1- and 100-fold over baseline, i.e. between gate 1 and gate 5, which represent the lowest and the highest levels of RFP fluorescence, and therefore AP2σ2, respectively. (B) Predicted linear regressions of mean EC50 with effects owing to dominant-negative and haploinsufficiency mutants. Loss-of-function mutants with dominant-negative effects on the WT protein will exert greater effects on EC50 values with increasing concentrations (red line), whereas loss-of-function mutants associated with haploinsufficiency of the WT protein will not affect the EC50 values with increasing concentrations as the EC50 will depend on the concentration of WT protein, which will remain constant (blue line). (C–E) Linear regression of the mean EC50 of cells with increasing levels of AP2σ2 expression demonstrate no significant deviation from zero in cells transfected with the WT, R15, C15 or L15 expression constructs (r² = 0.07, P = 0.67; r² = 0.82, P < 0.05; r² = 0.84, P < 0.05; r² = 0.99, P < 0.0001), thereby indicating that FHH3-associated AP2σ2 mutations may act in a dominant-negative manner. All experiments were conducted on N = 8 separate occasions.
between the expression levels of all three AP2o2 mutants and the mean cellular EC50 responses (Cys15 r² = 0.82, P < 0.05; His15 r² = 0.84, P < 0.05; Leu15 r² = 0.99, P < 0.0001), but that increased expression levels of wild-type AP2o2 had no effect on EC50 values (r² = 0.07, P = 0.67) (Fig. 6). These findings indicate a potential dominant-negative effect of the FHH3-causing Arg15 AP2o2 mutants.

Discussion

Our results, which have identified AP2S1 mutations that only result in missense substitutions of the Arg15 residue in 17 additional FHH probands, have helped to establish a genotype–phenotype correlation between AP2o2 mutants and the severity of hypercalcaemia; devise an index based on measurements of plasma calcium and magnesium concentrations and urinary clearances of calcium and creatinine to differentiate between FHH1 and FHH3; elucidate the occurrence of a mutational bias at the AP2o2 Arg15 residue in FHH3 patients and define the likely genetic mechanism for AP2o2 mutations as being a dominant-negative action in causing FHH3. In addition, our study shows that FHH3 can be associated with de novo AP2S1 mutations, consistent with a previous report (12), thereby indicating that patients may not have a family history of the disorder and that AP2S1 mutations may be associated with non-familial forms of hypercalcaemia and hypocalciuria.

The identification by this study of AP2S1 mutations in 17 additional probands with FHH3, together with ours and other previous reports (11,12,14), yields a total of 36 probands with such AP2o2 mutations, which all involve the Arg15 residue and result in 1 of 3 missense substitutions comprising Arg15Cys, Arg15His and Arg15Leu. Our analyses of these studies reveal genotyope–phenotype correlations for these AP2o2 mutations, in which the Arg15Leu mutation is associated with the most pronounced hypercalcaemia, whereas Arg15His is associated with the mildest increase in serum calcium concentrations. This is surprising and the mechanisms underlying these genotype–phenotype correlations are unclear, as three-dimensional modelling and in vitro functional expression studies indicate that all three Arg15 AP2S1 mutations disrupt CaSR activity to a similar extent (11), and the basis for the differences between our in vivo clinical observations and in vitro results remains to be elucidated.

Our study has also revealed phenotypic differences between FHH1 and FHH3. In particular, FHH3 was associated with significantly greater elevations of serum calcium, and >20% of FHH3 patients in this cohort had symptomatic hypercalcaemia. These findings are not typical of FHH, which is considered to be a benign and asymptomatic disorder, and in general associated with serum calcium concentrations that remain within 10% of the upper limit of the normal range (1). In addition to the more severe hypercalcaemia, FHH3 was associated with more pronounced suppression of urinary calcium excretion than FHH1. The more marked serum and urinary calcium phenotype of FHH3 contrasts with the results of in vitro studies that indicate FHH3-causing AP2S1 mutations to lead to a milder shift in the set point of CaSR-expressing cells when compared with FHH1-causing CASR mutations (11). These findings suggest that AP2o2 mutations may influence Ca²⁺ homeostasis through effects on cell membrane proteins other than the CaSR. Indeed, the renal thick ascending limb Na⁺/K⁺/2Cl⁻ (NCC2) transporter, which is pivotal for urinary calcium reabsorption, has been demonstrated to be regulated by clathrin-mediated endocytosis (24). Other phenotypic differences between FHH3 and FHH1 included the occurrence of low BMD and cognitive dysfunction in FHH3. Low BMD occurred in five FHH3 probands with AP2S1 mutations whereas patients with FHH1, owing to CASR mutations, have been reported to have normal bone resorption rates and BMD measurements at the spine, hip and forearm (25). Cognitive dysfunction was observed in seven FHH3 probands, four of which presented with serum calcium concentrations of >3.0 mmol/l, and exposure to marked hypercalcaemia in infancy or childhood may have led to developmental delay and adversely affected neurological development in these individuals (26). It is also possible that expression of mutant AP2o2 subunits within the brain may directly influence neurological development, consistent with the role of the AP2 complex in mediating receptor trafficking within neuronal synapses of the hippocampus (27), a region of the brain required for memory acquisition and spatial orientation. The Arg15Leu AP2S1 mutation was also associated with recurrent pancreatitis in one FHH3 proband, a finding consistent with FHH1, in which recurrent pancreatitis has occasionally been reported (28). However, FHH1 probands with pancreatitis typically harbour heterozygous mutations of both the CASR and serine protease inhibitor, Kazal type 1 (SPINK1) genes (29). In contrast, the affected FHH3 proband did not harbour a mutation of SPINK1 or other genes associated with pancreatitis (19), and this proband’s marked hypercalcaemia may have been sufficient to disrupt pancreatic function. Given these possible phenotypic differences between FHH3 and FHH1, we sought to design an index based on clinical biochemistry tests of serum and urine that would help to differentiate these two disorders. The findings that FHH3 is associated with greater elevations in serum calcium and magnesium concentrations, and reductions in urinary calcium excretion, when compared with FHH1, led us to design the calcium–magnesium–calcium clearance ratio (CMCR), calculated as sCa × sMg/100 × CCCR. Assessment of the performance characteristics of this index indicated that FHH patients with CMCR ≥5.0 were significantly more likely to have FHH3. Thus, the CMCR index may have utility in the clinical setting for directing FHH patients for either CASR or AP2S1 gene analysis, with patients having a CMCR value of ≥5.0 being prioritized for AP2S1 analysis (Fig. 7). However, ~30% of FHH3 patients have CMCR values that overlap with that of FHH1 patients (Fig. 3), and combined analysis of the CASR, AP2S1 and GNA11 genes is suggested in individuals with CMCR values of ≤5.0 (Fig. 7). No correlation was observed between the CMCR index and codon 15 genotype of the FHH3 patients. The CMCR index requires further validation in studies of other populations and using alternate biochemical assays.

The finding that all the 36 reported FHH3 patients, of which 17 are from this study, harbour either an Arg15Cys, Arg15His or Arg15Leu mutation, highlights the importance of this evolutionary conserved residue for AP2-mediated Ca²⁺ homeostasis (11,12,14). Furthermore, the absence of the other possible missense substitutions at this codon (Arg15Gly, Arg15Pro or Arg15Ser) indicates mutation bias at the AP2o2 Arg15 residue. Our in vitro characterization of the non-observed Gly15, Pro15 or Ser15 mutations demonstrated these mutant AP2o2 proteins to be expressed and to result in an impairment of CaSR activity. However, the Gly15, Pro15 or Ser15 mutants had additional deleterious effects that led to a reduction in the numbers of cells expressing these mutants. These findings indicate a potential role for the AP2 complex in cell growth or viability and are consistent with a previously reported study of mice harbouring a germline ablation of the AP2α2 subunit, which led to early embryonic lethality, thus highlighting the involvement of the ubiquitously expressed AP2 complex in mammalian development (30). Thus, a possible explanation for the absence of the Arg15Gly, Arg15Pro and Arg15Ser AP2o2 mutations in patients.
is that these deleterious mutations likely result in embryonic lethality, whereas the FHH3-causing AP2σ2 mutations Arg15Cys, Arg15His and Arg15Leu are observed in patients, as these mutations are not associated with deleterious effects on cell growth, but are tolerated and compatible with embryonic and postnatal survival.

Our findings of a likely dominant-negative effect of FHH3-causing Arg15 AP2σ2 mutations are consistent with previous mutagenesis studies of the AP2 complex (31). Indeed, overexpression of a mutated μ2-subunit (AP2μ2), which displayed diminished capacity to bind to some cargo proteins, has been reported to inhibit receptor trafficking in a dominant-negative manner (31). The precise mechanisms of such dominant-negative actions remain to be established but three possibilities are (1) the incorporation of a mutant e2 subunit may impair the assembly of the tetrameric complex, (2) incorporation of the mutant e2 subunit may alter the structure of the AP2 complex and hinder the efficiency of wild-type AP2α, AP2β and AP2μ subunits (32) and (3) the mutant AP2 complex may potentially sequester the CaSR at the plasma membrane and prevent wild-type AP2 molecules from effectively trafficking this GPCR.

In summary, our studies of AP2S1 mutations, which highlight FHH3 as a distinct disorder of Ca\(^{2+}\) homeostasis and demonstrate the importance of the AP2α2 Arg15 residue for AP2 function, have revealed genotype–phenotype correlations with mutation bias at the Arg15 residue, as well as a likely dominant-negative mechanism of action.

**Materials and Methods**

**Subjects**

Informed consent was obtained from individuals, using protocols approved by the local and national ethics committees (MREC/02/
2/93). Sixty-five unrelated probands (22 males and 43 females) were ascertained. The age at diagnosis or presentation ranged from early infancy to 76 years, and all probands had hypercalcaemia with normal or elevated PTH concentrations, in association with inappropriately low urinary calcium excretion. Previous mutational analysis of CASR and GNA11 had not identified any abnormalities of the coding regions and exon-intron boundaries of these genes (8,9).

DNA sequence analysis

Leukocyte DNA was extracted from venous blood samples and quantified using the High sensitivity Qubit system (Invitrogen) (11). AP2S1-specific primers were used to perform PCR amplification of the five exons and eight intron-exon boundaries of the AP2S1 gene, as previously reported (11). DNA sequence analysis of the PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and an ABI automated capillary sequencer (Applied Biosystems), as reported (8). DNA sequence abnormalities and co-segregation in families were confirmed by restriction endonuclease analysis (New England Biolabs), as described (11).

Computer modelling of the AP2 structure

The crystal structure of the AP2 heterotetramer bound to an acidic dileucine peptide has been reported previously (18). The PyMOL Molecular Graphics Systems (version 1.2r3pre, Schrödinger) was used to model the effect of the FHH3-associated AP2α2 mutations on the interaction with the acidic dileucine motif peptide using the three-dimensional structure of AP2α2 archived in the Protein Data Bank at the European Bioinformatics Institute with the accession number 2JKR (8,9,11,18). The effect of the Arg15Cys, Arg15Leu, Arg15His, Arg15Pro, Arg15Ser and Arg15Gly mutations on the interaction of AP2 with the acidic dileucine peptide was modelled using PyMod plug-in and Modeller.21 (9).

Generation of AP2S1 expression constructs

An AP2α2 expression construct was generated by cloning the full-length AP2S1 coding region into the bidirectional cloning vector pBI-CMV4-RFP (Clontech), which allows for co-expression of AP2α2 and RFP at equivalent levels (11,33). The use of RFP minimized overlap between the emission spectra of this reporter gene and the indo-1-AM Ca²⁺-binding fluorophore. AP2S1 mutations were introduced into the construct by site-directed mutagenesis (QuickChange Lightning, Stratagene) and confirmed by DNA sequence analysis, as described (8,11).

Cell culture and transfection

Functional studies of AP2S1 mutations were performed in HEK293 cells that stably expressed the CaSR (11). HEK293 cells were used because suitable parathyroid and renal tubular cells are not available, and HEK293 cells have been established as a model for such studies (8,9,11,13). The reported stably CaSR-transfected HEK293 cell line (HEK-CaSR) (9,11) was cultured in high-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 1% genetin (11). A high level of CaSR expression in these cells was confirmed by western blot analysis of whole-cell protein extract using a mouse monoclonal antibody to human CaSR (ADD; Abcam, ab19347, 1:1000) (9,11). The wild-type and mutant AP2α2 constructs were transiently transfected into HEK-CaSR cells using Lipofectamine 2000 (Invitrogen) (9,11). Expression of RFP was used as a surrogate for AP2α2 expression as it is expressed at equivalent levels to AP2α2 by the pBI-CMV4-RFP bidirectional vector (11). Western blot analysis of cellular protein extract was undertaken using a rabbit polyclonal antibody to RFP (Thermo Scientific, PA1-986, 1:500) (11). The membrane was re-probed with mouse anti-GAPDH antibody (Abcam, ab8245, 1:3000) as a loading control. Successful transfection was also confirmed by visualising RFP fluorescence using an Eclipse E400 fluorescence microscope with an epifluorescence filter, and images were captured using a DXM1200C digital camera and NIS Elements software (Nikon) (9,11). The effect of mutant AP2α2 proteins on cell growth was assessed by determining the percentage increase in cell numbers over a 24-h period, as follows: Twenty-four hours after transfection, equal numbers of cells were seeded into a 96-well plate, and the cells were imaged, at 48 and 72 h, using fluorescence microscopy, as mentioned above (9,11). Following blinding to both transfection and time point, the numbers of transfected cells were counted manually at 48 and 72 h post-transfection, and the percentage increase in cells determined using Microsoft Excel and statistical analysis undertaken in GraphPad Prism (GraphPad) (9,11).

Measurement of Ca²⁺ responses

The effect of mutant AP2α2 proteins on CaSR-mediated Ca²⁺ responses were assessed by determining EC₅₀ values (i.e. [Ca²⁺]₀ required for 50% of the maximal response) and comparing these to the wild-type EC₅₀, as reported (8,9,11). Briefly, 48 h after transfection, the cells were harvested, washed in calcium- and magnesium-free Hank’s balanced salt solution (HBSS) (Invitrogen) and loaded with 1 μM indo-1-acetoxyxymethylster (Indo-1-am) (Molecular Probes) for 1 h at 37 °C (8,9,11). After the removal of free dye, the cells were resuspended in calcium- and magnesium-free HBSS and maintained at 37°C. Flow cytometry was performed with a Beckman Coulter MoFlo XDP equipped with JDSU Xcyte UV Laser and a Coherent Sapphire 488 Laser using a 550LP dichroic mirror and 580/30 bandpass filter. Single cells were isolated from debris on the basis of morphology using forward scatter and side scatter readings (8,9,11). Cells were stimulated by sequentially adding calcium to the calcium- and magnesium-free HBSS to progressively increase the [Ca²⁺]₀ from 0 to 15 mM. The baseline fluorescence ratio was measured for 2 min, the fluorescence ratio compared with the time was recorded and data were collected for 2 min at each [Ca²⁺]₀. Cytometry Summit software was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus expressed as a normalized response (8,9,11). Analysis of five separate populations of cells gated for increasing RFP as a concordant surrogate for increasing AP2α2 expression was undertaken and concentration–response curves generated using the normalized response at each of nine different [Ca²⁺]₀ (0–15 mM) for each separate experiment. Nonlinear regression of the concentration–response curves was performed with GraphPad Prism (GraphPad) to calculate the EC₅₀ for each separate experiment (8,11). Subsequent linear regression of the mean EC₅₀ (N = 8 experiments) for each AP2S1-pBI-CMV4-RFP expression vector was undertaken with GraphPad Prism (GraphPad) (9,11).

Statistical analyses

The phenotypic data from this study were pooled with those of our previous studies of FHH3 patients and families (11,20,23). To undertake a comparison of phenotypes between FHH3 and FHH1, we used data from 43 previously reported unrelated...
FHH1 probands (13 males and 30 females, aged 1–84 years) who had germline heterozygous CASR mutations (8). Comparisons of continuous variables between two groups were undertaken using the Mann–Whitney U test, and the Kruskal–Wallis test was used to compare multiple groups. Categorical variables were analysed using the Chi-squared test. ROC analysis using values of the CMCR sensitivity and specificity was performed to study the discriminative power of the CMCR index to distinguish between FHH1 and FHH3. Comparisons of EC50 values were performed using the F test, as described (9,11). The Mann–Whitney U test was used to compare the proliferation rates of cells expressing wild-type or mutant AP2α2 proteins. All analyses were undertaken using GraphPad Prism (GraphPad) and are presented as mean ± SEM unless otherwise stated. A value of P < 0.05 was considered significant for all analyses.

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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