A Novel Albumin Gene Mutation (R222I) in Familial Dysalbuminemic Hyperthyroxinemia

Nadia Schoenmakers,* Carla Moran,* Irene Campi, Maura Agostini, Olivia Bacon, Odelia Rajanayagam, John Schwabe, Timothy Barrett, Frank Geoghegan, Maralyn Druce, Paolo Beck-Peccoz, Angela O’Toole, Penelope Clark, Michelle Bignell, Greta Lyons, David Halsall, Mark Gurnell, and Krishna Chatterjee

University of Cambridge Metabolic Research Laboratories (N.S., C.M., M.A., O.R., G.L., M.G., K.C.), Wellcome Trust-Medical Research Council Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge CB2 0QQ, United Kingdom; Institute of Endocrine Sciences (I.C., P.B.-P.), University of Milan, 20132 Milan, Italy; Department of Biochemistry (J.S.), University of Leicester, Leicester LE1 7RH, United Kingdom; Departments of Paediatrics (T.B.) and Clinical Chemistry (M.B.), Birmingham Childrens Hospital, Birmingham B15 2TT, United Kingdom; Departments of Endocrinology (M.D.), St Bartholomew’s Hospital, London E1 4NS, United Kingdom; Department of Clinical Biochemistry (F.G.), Ealing Hospital, London UB1 3EU, United Kingdom; Department of Clinical Biochemistry (A.O., P.C.), Selly Oak Hospital, Birmingham B29 6JD, United Kingdom; and Department of Clinical Biochemistry (O.B., S.B., D.H.), Addenbrooke’s Hospital, Cambridge CB2 20O, United Kingdom

Context: Familial dysalbuminemic hyperthyroxinemia, characterized by abnormal circulating albumin with increased T4 affinity, causes artefactual elevation of free T4 concentrations in euthyroid individuals.

Objective: Four unrelated index cases with discordant thyroid function tests in different assay platforms were investigated.

Design and Results: Laboratory biochemical assessment, radiolabeled T4 binding studies, and ALB sequencing were undertaken. 125I-T4 binding to both serum and albumin in affected individuals was markedly increased, comparable with known familial dysalbuminemic hyperthyroxinemia cases. Sequencing showed heterozygosity for a novel ALB mutation (arginine to isoleucine at codon 222, R222I) in all four cases and segregation of the genetic defect with abnormal biochemical phenotype in one family. Molecular modeling indicates that arginine 222 is located within a high-affinity T4 binding site in albumin, with substitution by isoleucine, which has a smaller side chain predicted to reduce steric hindrance, thereby facilitating T4 and rT3 binding. When tested in current immunoassays, serum free T4 values from R222I heterozygotes were more measurably abnormal in one-step vs two-step assay architectures. Total rT3 measurements were also abnormally elevated.

Conclusions: A novel mutation (R222I) in the ALB gene mediates dominantly inherited dysalbuminemic hyperthyroxinemia. Susceptibility of current free T4 immunoassays to interference by this mutant albumin suggests likely future identification of individuals with this variant binding protein. (J Clin Endocrinol Metab 99: E1381–E1386, 2014)
with enhanced T₄ binding affinity. An arginine-to-histidine mutation at residue 218 (R218H) was first described (2, 3) and is the most common causal variant in Caucasians but also recognized in Hispanic/Puerto Rican (4) and Chinese (5) cases. Substitution of proline for arginine at the same codon (R218P), resulting in markedly elevated T₄ concentrations, has been described in Japanese and Swiss subjects (6, 7). A third albumin mutation (L66P), identified in a Thai kindred, is associated with predominant elevation of T₃ concentrations (8).

Here we describe a novel, heterozygous ALB defect, with substitution of isoleucine for arginine at codon 222 (R222I) in three African (Somali) subjects and one East European (Croatian) family, identified on the basis of discrepant thyroid function tests, with hyperthyroxinemia. Enhanced T₄ binding to this albumin variant correlates with molecular modeling showing that this amino acid change likely reduces steric hindrance within its high-affinity T₄ binding pocket. Elevated free T₄ measurements in most commonly used immunoassay platforms suggests that additional cases harboring this novel FDH variant will be identified.

**Patients and Methods**

**Methods**

All investigations were part of an ethically approved protocol and/or clinically indicated, being undertaken with the consent from patients and/or next of kin.

**Biochemical measurements**

Thyroid hormones [free T₄ (FT₄) and free T₃] and TSH were measured using automated immunoassay systems (Advia Centaur; Siemens; Wallac DELFIA Ultr; PerkinElmer; Access; Beckman-Coulter; Elecsys; Roche Diagnostics; Architect; Abbott Diagnostics). T₄ binding globulin (TBG) was measured by immunoassay (Siemens Immulite). Equilibrium dialysis FT₄ was measured by RIA (Quest Diagnostics). Total T₃ and rT₃ were measured using automated immunoassay (Siemens Immulite). Equilibrium dialysis was normal (Table 1). Hence, an albumin protein abnormality was investigated for low weight, was found to have elevated FT₄ but unsuppressed TSH (Table 1). His mother and two siblings exhibited similarly abnormal thyroid function tests [mother: FT₄ 36.9 pmol/L (reference range) [RR] 10–24], TSH 1.57 mU/L (0.5–5.0); sibling 1: FT₄ 30.9 pmol/L (RR 11–22), TSH 2.01 mU/L (RR 0.4–3.5); sibling 2: FT₄ 48.5 (12–25), TSH 3 mU/L (RR 0.4–3.5)]. Proband 2 was an unrelated 41-year-old Somali male (P2) and was referred with a similar biochemistry (Table 1).

**Molecular modeling**

The R222I mutant albumin was modeled (Pymol) using previously described wild-type albumin (1bm0) albumin-T₄ (1hk1), R218H FDH mutant albumin-T₄ (1hk2), and R218P FDH mutant albumin-T₄ (1hk3) crystal structures (11), selecting the rotamer with the fewest clashes.

**Results**

**Clinical and biochemical features**

Proband 1 was a 2.5-year-old, Somali boy (P1), investigated for low weight, was found to have elevated FT₄ but unsuppressed TSH (Table 1). His mother and two siblings exhibited similarly abnormal thyroid function tests [father: FT₄ 41.2 pmol/L (RR 10–22), TSH 3.2 mU/L (RR 0.28–4.3); sibling 1: FT₄ 33.5 pmol/L (RR 10–22), TSH 3.0 mU/L (RR 0.28–4.3)].

Although local testing in all probands showed markedly raised FT₄ concentrations, FT₄ measurements using the two-step DELFIA method were quite discordant, being near normal; furthermore, FT₄ measured by equilibrium dialysis was normal (Table 1). These observations suggested analytical interference with FT₄ measurement, with diagnostic possibilities including abnormal circulating thyroid hormone binding proteins. Although total T₄ was raised in each proband, the TBG levels were normal (Table 1). Hence, an albumin protein abnormality was considered. Serum binding of ¹²⁵I-T₄ in each proband was markedly raised (Table 1), comparable with values (28%–44%) in sera from known FDH cases, harboring the R218H albumin mutation. Gel electrophoresis of serum from an affected individual identified excess ¹²⁵I-T₄ binding to albumin [Figure 1A, panel (iii)]. The abnormal electrophoretic profile was similar to the pattern of ¹²⁵I-T₄ binding in serum from a known FDH case, harboring the R218H albumin mutation [Figure 1A, panel (ii)].

**Molecular genetic studies**

ALB sequencing of probands (P1-P4) revealed heterozygosity for a single-nucleotide substitution (AGA
to ATA), corresponding to an arginine to isoleucine change at codon 222 in the predicted protein sequence, with no other coding region changes. The mutation is not present in 100 control DNA samples and normal genome data sets (dBSNP, 1000 Genomes) including more than 2000 African-American alleles (Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, WA, http://evs.gs.washington.edu/EVS/, May 11, 2013). Genotyping for single-nucleotide polymorphisms around ALB indicates that the Somali cases share an extended haplotype, suggesting common ancestry, whereas the mutation occurs on a different haplotype background in Caucasian proband 4 (Supplemental Figure 1).

The mother and siblings of P1, with abnormal thyroid function tests, were also heterozygous for this nucleotide change. The ALB mutation cosegregated with phenotype in family members of P4, being present in individuals (father and brother) with elevated FT4 results and serum 125I-T4 binding and absent in her unaffected mother with normal FT4 concentrations and radiolabeled hormone binding (Supplemental Figure 2).

**FT4, T3, and rT3 measurements in affected cases**

The index cases were identified on the basis of discordant FT4 results using one-step (Roche Elecsys or Siemens Immulite) hormone assays. To investigate the performance of commonly used assay platforms, FT4 concentrations were measured using sera from ALB R222I heterozygotes in different two-step [(DELFIA Ultra (PerkinElmer), Architect (Abbot Diagnostics), Access (Beckman Coulter)] and one-step [Advia Centaur (Siemens Medical Diagnostics); Elecsys E170 (Roche)] immunoassays (Figure 1B). Affected individuals exhibited a similar pattern, with FT4 measurements being more elevated in one-step (Centaur, Elecsys) than two-step (Architect, Delfia) platforms; exceptionally, FT4 values were most markedly raised with the two-step Beckman Access method.

We assayed total T3 and rT3 by tandem mass spectrometry using ALB R222I heterozygote sera and compared concentrations with R218H ALB FDH cases. Total T3 concentrations were slightly raised in two R222I ALB cases but normal in all other subjects with either variant albumin (Supplemental Figure 3). In contrast, rT3 concentrations were markedly elevated in ALB R222I sera, being 40- to 70-fold elevated, but were normal or only marginally raised (1.1-fold) in R218H ALB FDH cases (Figure 1C). Such elevation was also seen when rT3 was measured by immunoassay in sera from R222I FDH cases (rT3/H11022 ng/mL, normal range 0.11–0.32 ng/mL). rT3 displaced 125I-T4 binding to R222I ALB sera much more readily than in control or R218H FDH cases (Supplemental Figure 4).

**Molecular modeling of R222I mutant albumin**

In the T4-albumin crystal structure, T4 interacts with side chains of three residues (R218, W214, and R222) within a high-affinity binding site. Comparison with an unoccupied protein structure shows that T4 binding requires significant rearrangement of these three side chains

| Table 1. Biochemical Measurements in Index Cases |
|--------------------------------------------------|
| *TSH, mU/L* |
| **Platform** | **Reference range** |
| Immulite 2000 | 0.3–4.0 |
| Immulite 2000 | 0.3–4.0 |
| Roche Elecsys | 0.27–4.2 |
| Roche Elecsys | 0.3–4.0 |
| **FT4, pmol/L** |
| **Platform** | **Reference range** |
| Immulite 2000 | 12–25 |
| DELFIA | 21.8 |
| DELFIA | 9–20 |
| DELFIA | 9–20 |
| **FT4 by equilibrium dialysis, ng/dL** |
| **Platform** | **Reference range** |
| Quest | 0.8–2.7 |
| Roche Elecsys | 12–25 |
| DELFIA | 273 |
| DELFIA | 9–20 |
| **Total T4, nmol/L** |
| **Platform** | **Reference range** |
| DELFIA | 69–141 |
| Immulite | 20.3 |
| Immulite | 14–31 |
| Cisbio | 11.3–28.9 |
| Immulite | 14–31 |
| **Radiolabeled T4 binding to serum** |
| **Platform** | **Reference range** |
| Immulite | <20% |
| Immulite | <20% |
| Immulite | Increased |
| Immulite | 38% |
| Immulite | 49% |

Numbers in bold denote that they are outside the reference range. Abbreviation: ND, not done.

* Varying reference data for the same assay platform reflect differing normal ranges used by local laboratories.

* Exact percentage binding unavailable.
Figure 1. Biochemical studies in FDH cases and molecular modeling of albumin mutation. A, Electrophoregrams showing binding of $^{125}$I-T$_4$ to serum proteins in serum containing an albumin mutation (R218H) known to confer FDH [left panel, (i)] and an individual with hyperthyroxinemia (ii) R222I.

B, Comparison of thyroxine binding capacity (FT$_4$) in different assays: Centaur, Elecsys, Access, Architect, DELFIA.

C, Reverse T$_3$ levels for R218H and R222I mutants.

D, Molecular modeling of albumin mutants R222I and T3.
(Figure 1D, left panel). Substitution of arginine at codon 222 by isoleucine reduces steric hindrance, enhancing T₄ binding (Figure 1D, middle panel). Iodines in the inner ring of T₄ are in close contact with side chains of R222 and W214. Superimposition of rT₃ (Figure 1D, right panel) with T₄, reveals that the absence of an inner ring iodine would provide more space in the pocket, with both the isoleucine 222 and tryptophan 214 imposing less steric hindrance; in contrast, substitutions at R218 are not predicted to influence rT₃ binding.

Discussion

Six individuals from three unrelated families of East African and three subjects of Caucasian East European origin were found to have euthyroid hyperthyroxinemia and nonsuppressed TSH concentrations, with assay-dependent discordant FT4 measurements suggesting analytical interference. Normal circulating TBG concentrations to-gether with increased radiolabeled ¹²⁵I-T₄ binding to serum or albumin from these cases suggested an ALB abnormality. Affected individuals are heterozygous for a missense ALB mutation (R222I); in one kindred, in which family members were available, heterozygosity for this ALB mutation segregates with both abnormal thyroid biochemical and ¹²⁵I-T₄ binding phenotypes.

The high-affinity binding site for T₄ in albumin contains three residues (R218, R222, and W214) whose side chains undergo marked displacement to accommodate T₄ binding (11). Consistent with this structural observation, substitution of histidine or proline with smaller side chains for arginine 218 likely reduces steric hindrance, explaining enhanced T₄ binding of these mutant proteins (11, 12). Likewise, modeling predicts that substitution of isoleucine for arginine 222, as occurs in our cases, also reduces steric hindrance. Indeed, an artificial albumin mutant (R222M), with a methionine residue with smaller side chain replacing R222, exhibits increased T₄ binding (13).

Our results suggest that, in general, one-step FT4 immunoassay methods are more susceptible to interference than R222I FDH sera and normal in R218H FDH cases; this finding is in accord with T₃ concentrations being raised in only 12% of R218H FDH (3). In contrast, total rT₃ concentrations were uniformly and more strikingly elevated in R222I sera than R218H FDH cases. Previously, raised rT₃ was documented in 50% of R218H FDH cases (8) and the R218H mutant albumin binds rT₃ with increased affinity (13). Because total hormone levels likely reflect hormone interaction with albumin in subjects with otherwise normal TH binding proteins, we hypothesized that rT₃ binding to R222I mutant albumin is enhanced, and competition assays with radiolabeled T₄ confirmed this. Structural modeling suggests a basis for this, with the absence of an inner ring iodine in rT₃, likely to further diminish steric hindrance from side chains of residues (Ile 222, Trp 214) which are in closest proximity to the inner ring iodines. The biochemical pattern of raised T₄, normal T₃, and elevated rT₃ concentrations in R222I FDH resembles that seen in patients after amiodarone exposure (16), raising the possibility that this genetic form of FDH might be confused with other clinical diagnostic possibilities.

In summary, we have identified a novel, heterozygous ALB mutation (R222I) in subjects of both East African and Caucasian Eastern European origin. R222I heterozygote sera exhibit a biochemical profile of elevated FT4 concentrations in many current immunoassay platforms, suggesting that this genetic cause of dysalbuminemic hyperthyroxinemia will be readily identified, perhaps in other populations.

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Figure 1 Continued. and elevated radiolabeled T₄ binding to serum containing an R222I albumin mutation (right panel, (ii)). B, FT4 measured by various one-step or two-step immunoassays in sera from different cases containing the R222I mutant albumin protein. C, rT₃ measured by liquid chromatography and tandem mass spectrometry in sera from R218H and R222I mutation cases. D, Crystallographic modeling of T₄ to the high-affinity binding site in subdomain IIA of the albumin molecule, illustrating the steric constraints imposed on T₄ binding. The left panel is a composite, showing the positions (in yellow) of the side chains of W214, R218, and R222 in the albumin structure not bound to T₄. When R222 is replaced by isoleucine (middle panel, in orange), the shorter side chain presents less steric hindrance to T₄ binding. rT₃ binding to R222I mutant albumin is also likely to be enhanced (right panel) because the loss of the inner iodine will further relieve steric hindrance with side chains of residues at positions 222 and 214.
Address all correspondence and requests for reprints to: V Krishna K. Chatterjee, Metabolic Research Laboratories, Wellcome Trust–Medical Research Council Institute of Metabolic Science, University of Cambridge, Level 4, Box 289, Addenbrooke’s Hospital, Cambridge CB2 0QQ, United Kingdom. Email: kkc1@medschl.cam.ac.uk.

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