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

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Context: Familial dysalbuminemic hyperthyroxinemia, characterized by abnormal circulating albumin with increased T_4 affinity, causes artefactual elevation of free T_4 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 T_4 binding studies, and *ALB* sequencing were undertaken. ^{125}I-T_4 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 T_4 binding site in albumin, with substitution by isoleucine, which has a smaller side chain predicted to reduce steric hindrance, thereby facilitating T_4 and rT_3 binding. When tested in current immunoassays, serum free T_4 values from R222I heterozygotes were more measurably abnormal in one-step vs two-step assay architectures. Total rT_3 measurements were also abnormally elevated.

Conclusions: A novel mutation (R222I) in the *ALB* gene mediates dominantly inherited dysalbuminemic hyperthyroxinemia. Susceptibility of current free T_4 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)**

Familial dysalbuminemic hyperthyroxinemia (FDH), the most common heritable cause of elevated total T_4 levels in euthyroid subjects, has an estimated prevalence of 1 in 10 000 individuals (1). Consistent with its dominant inheritance, the disorder is associated with heterozygous albumin (*ALB*) gene defects, generating mutant proteins

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Abbreviations: ALB, albumin; FDH, familial dysalbuminemic hyperthyroxinemia; FT4, free T_4; RR, reference range; TBG, T_4 binding globulin.

with enhanced T_4 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 T_4 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 T_3 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 T_4 binding to this albumin variant correlates with molecular modeling showing that this amino acid change likely reduces steric hindrance within its high-affinity T_4 binding pocket. Elevated free T_4 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 T_4 (FT4) and free T_3] and TSH were measured using automated immunoassay systems (Advia Centaur; Siemens; Wallac DELFIA Ultr; PerkinElmer; Access; Beckman-Coulter; Elecsys; Roche Diagnostics; Architect; Abbott Diagnostics). T_4 binding globulin (TBG) was measured by immunoassay (Siemens Immulite). Equilibrium dialysis FT4 was measured by RIA (Quest Diagnostics). Total T_3 and rT_3 were measured in deproteinized samples by separation using C18 column chromatography followed by electrospray mass spectrometry or (rT_3 in a subset of cases) by competitive RIA (Quest Diagnostics).

Radiolabeled T_4 binding studies and gel electrophoresis

Serum binding of ^{125}I-T_4 was assayed with excess cold T_4 to saturate binding sites on TBG as described previously (9); inclusion of cold rT_3 in this assay enabled comparison of its binding with R218H and R222I albumin mutants. ^{125}I-T_4 binding to serum proteins was analyzed by gel electrophoresis (Mayo Medical Laboratories) as described previously (10).

Albumin gene sequencing

Exons of the human albumin gene were PCR amplified from genomic DNA using specific primers (listed in Supplemental Material) and analyzed by Sanger sequencing.

Molecular modeling

The R222I mutant albumin was modeled (Pymol) using previously described wild-type albumin (1bm0) albumin-T_4 (1hk1), R218H FDH mutant albumin-T_4 (1hk2), and R218P FDH mutant albumin-T_4 (1hk3) crystal structures (11), selecting the rotamer with the fewest clashes.

Results

Clinical and biochemical features

Proband 1 was a 2.5-year-old, Somalian 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 [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). Proband 3 was a 20-year-old Somali female (P3), investigated for fatigue and weight gain, and showed hyperthyroxinemia with nonsuppressed TSH (Table 1). Proband 4 was a 22-year-old Caucasian female (P4) from Croatia, investigated for asthenia and anxiety, and was found to have hyperthyroxinemia with nonsuppressed TSH. Her sibling and father exhibited similar thyroid function tests [father: FT4 41.2 pmol/L (RR 10–22), TSH 3.2 mU/L (RR 0.28–4.3); sibling: FT4 33.5 pmol/L (RR10–22), 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 T_4 was raised in each proband, the TBG levels were normal (Table 1). Hence, an albumin protein abnormality was considered. Serum binding of ^{125}I-T_4 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 ^{125}I-T_4 binding to albumin [Figure 1A, panel (ii)]. The abnormal electrophoretic profile was similar to the pattern of ^{125}I-T_4 binding in serum from a known FDH case, harboring the R218H albumin mutation [Figure 1A, panel (i)].

Molecular genetic studies

ALB sequencing of probands (P1-P4) revealed heterozygosity for a single-nucleotide substitution (AGA

Table 1. Biochemical Measurements in Index Cases

	Proband 1	Proband 2	Proband 3	Proband 4
TSH, mU/L	1.89	1.4	1.71	3.6
Platform	Immulite 2000	Roche Elecsys	Roche Elecsys	Roche Elecsys
Reference range[a]	0.3–4.0	0.27–4.2	0.3–4.0	0.28–4.3
FT4, pmol/L	**35.3**	**50.9**	**39.3**	**37**
Platform	Immulite 2000	Roche Elecsys	Roche Elecsys	Roche Elecsys
Reference range[a]	12–25	12–22	9–20	10–22
FT4, pmol/L	**21.8**	**22**	**20.7**	16
Platform	DELFIA	DELFIA	DELFIA	DELFIA
Reference range	9–20	9–20	9–20	9–20
FT4 by equilibrium dialysis, ng/dL	2.2	ND	2.1	1.7
Platform	Quest	ND	Quest	Quest
Reference range[a]	0.8–2.7	ND	1.0–2.4	0.8–2.7
Total T_4, nmol/L	**303**	**273**	**275**	**204**
Platform	DELFIA	DELFIA	DELFIA	DELFIA
Reference range	69–141	69–141	69–141	69–141
TBG, μg/mL	20.3	20.5	20.6	16.5
Platform	Immulite	Cisbio	Immulite	Immulite
Reference range	14–31	11.3–28.9	14–31	14–31
Radiolabeled T_4 binding to serum	37%	Increased[b]	38%	49%
Reference range	<20%	<20%	<20%	<20%

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

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

[b] Exact percentage binding unavailable.

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 ^{125}I-T_4 binding and absent in her unaffected mother with normal FT4 concentrations and radiolabeled hormone binding (Supplemental Figure 2).

FT4, T_3, and rT_3 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 T_3 and rT_3 by tandem mass spectrometry using ALB R222I heterozygote sera and compared concentrations with R218H ALB FDH cases. Total T_3 concentrations were slightly raised in two R222I ALB cases but normal in all other subjects with either variant albumin (Supplemental Figure 3). In contrast, rT_3 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 rT_3 was measured by immunoassay in sera from R222I FDH cases ($rT_3 > 2$ ng/mL, normal range 0.11–0.32 ng/mL). rT_3 displaced ^{125}I-T_4 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 T_4-albumin crystal structure, T_4 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 T_4 binding requires significant rearrangement of these three side chains

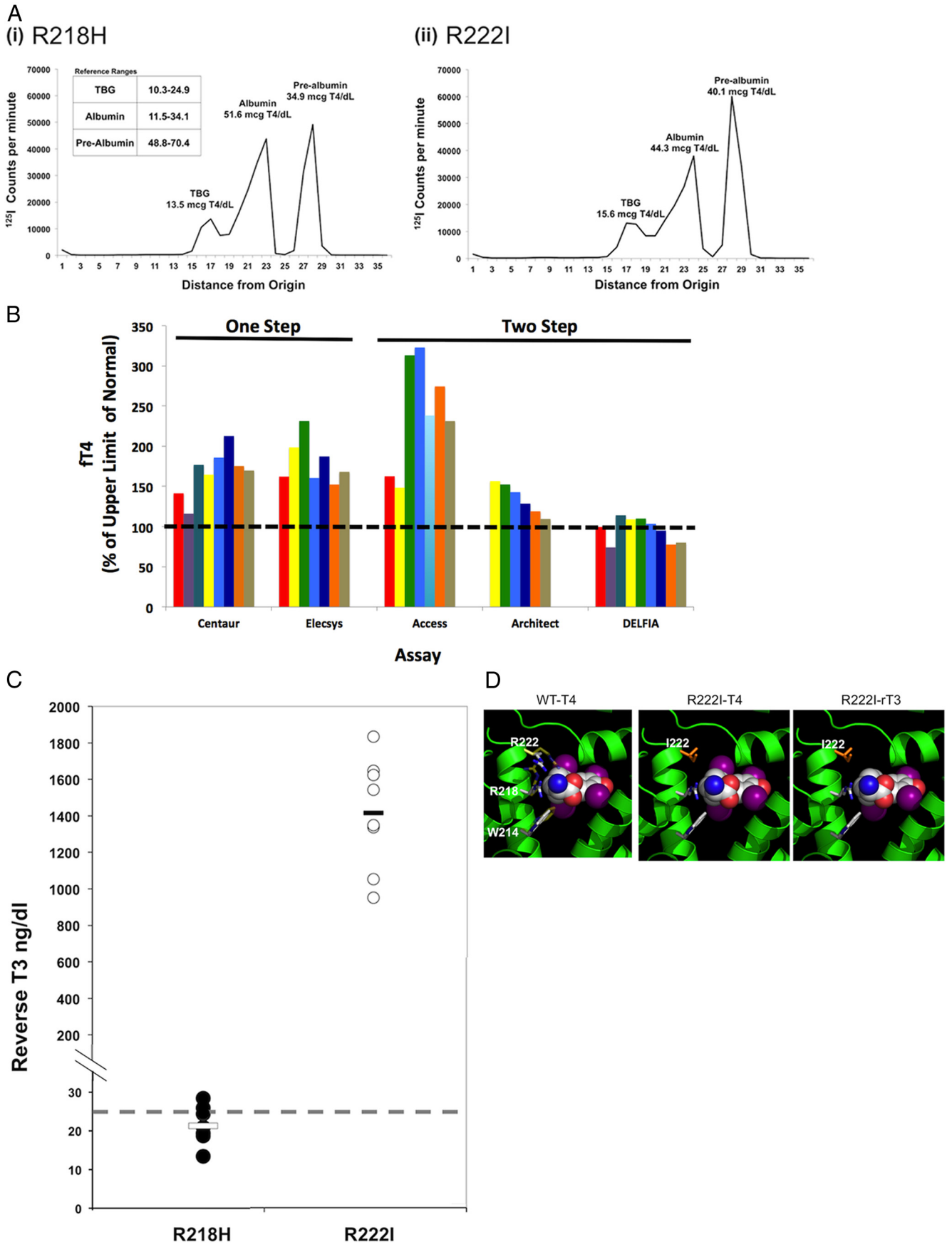

Figure 1. Biochemical studies in FDH cases and molecular modeling of albumin mutation. A, Electrophoregrams showing binding of ^{125}I-T_4 to serum proteins in serum containing an albumin mutation (R218H) known to confer FDH [left panel, (i)] and an individual with hyperthyroxinemia

(Figure 1D, left panel). Substitution of arginine at codon 222 by isoleucine reduces steric hindrance, enhancing T_4 binding (Figure 1D, middle panel). Iodines in the inner ring of T_4 are in close contact with side chains of R222 and W214. Superimposition of rT_3 (Figure 1D, right panel) with T_4, 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 rT_3 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 together with increased radiolabeled ^{125}I-T_4 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 ^{125}I-T_4 binding phenotypes.

The high-affinity binding site for T_4 in albumin contains three residues (R218, R222, and W214) whose side chains undergo marked displacement to accommodate T_4 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 T_4 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 T_4 binding (13).

Our results suggest that, in general, one-step FT4 immunoassay methods are more susceptible to interference by R222I FDH sera than two-step designs. This pattern resembles differential susceptibility of such assays with R218H FDH sera (14), presumably reflecting the interaction of labeled T_4 analogs with albumin in one-step assays, which does not occur in two-step or back-titration methods. It has been suggested that incubation buffer composition in the Beckman Access assay promotes T_4-albumin interaction, making this two-step method unexpectedly susceptible to interference (15).

Total T_3 concentrations were raised in two R222I FDH sera and normal in R218H FDH cases; this finding is in accord with T_3 concentrations being raised in only 12% of R218H FDH (3). In contrast, total rT_3 concentrations were uniformly and more strikingly elevated in R222I sera than R218H FDH cases. Previously, raised rT_3 was documented in 50% of R218H FDH cases (8) and the R218H mutant albumin binds rT_3 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 rT_3 binding to R222I mutant albumin is enhanced, and competition assays with radiolabeled T_4 confirmed this. Structural modeling suggests a basis for this, with the absence of an inner ring iodine in rT_3, 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 T_4, normal T_3, and elevated rT_3 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 T_4 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, rT_3 measured by liquid chromatography and tandem mass spectrometry in sera from R218H and R222I mutation cases. D, Crystallographic modeling of T_4, bound to the high-affinity T_4-binding site in subdomain IIA of the albumin molecule, illustrating the steric constraints imposed on T_4 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 T_4, superimposed on these displaced side chains (white) in the structure of albumin bound to T_4. When R222 is replaced by isoleucine (middle panel, in orange), the shorter side chain presents less steric hindrance to T_4 binding. rT_3 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.

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