DIO2 Thr92Ala Reduces Deiodinase-2 Activity and Serum-T3 Levels in Thyroid-Deficient Patients

Maria Grazia Castagna,[1] Monica Dentice,[2] Silvia Cantara,[1] Raffaele Ambrosio,[3] Fabio Maino,[1] Tommaso Porcelli,[2] Carlotta Marzocchi,[1] Corrado Garbi,[4] Furio Pacini,[1] and Domenico Salvatore[2,5]

[1]Department of Medicine, Surgery and Neuroscience, University of Siena, 53100 Siena, Italy; [2]Department of Clinical Medicine and Surgery, University of Naples "Federico II", 80131 Naples, Italy; [3]Istituto Di Ricovero e Cura a Carattere Scientifico SDN, 80143 Naples, Italy; [4]Dipartimento di Medicina Molecolare e Biotecnologie Mediche University Federico II, 80131 Naples, Italy; and [5]CEINGE–Biotecnologie Avanzate Scarl, 80145 Naples, Italy

Context: A substantial proportion of athyreotic levothyroxine (LT4)-treated patients experience hypothyroid-like symptoms. During LT4 replacement, levels of the active hormone triiodothyronine (T3) strictly depend on type 2-deiodinase (D2)-mediated activation of LT4. The Thr92Ala polymorphism and the 258 G/A in the *DIO2* gene have been associated with various clinical conditions.

Objectives: To investigate the effects of *DIO2* polymorphisms in thyroid hormone homeostasis.

Design: We compared the presurgical hormonal status of thyroidectomized LT4-treated patients who had a similar thyroid-stimulating hormone (TSH) level with their postsurgery status and analyzed their *DIO2* genotype in a subgroup of 102/140 (72.8%) of patients. We measured the enzymatic properties of Thr92Ala in living cells and in relevant generated mouse models.

Subjects and methods: A total of 140 thyroidectomized subjects were included. Serum free T3 (FT3), free thyroxine, and TSH levels were directly measured. Immunohistochemistry and immunoblotting were performed for D2 protein.

Results: The *DIO2* genotyping revealed an association between low FT3 values and Thr92Ala. Specifically, the mean postsurgery FT3 levels were significantly lower in patients carrying the mutated allele(s) than in wild-type patients, in whom FT3 postsurgical levels were similar to presurgery levels. The −258 G/A variation was not associated with hormonal alteration. We found that endogenous wild-type D2 and Thr92Ala share the same subcellular localization but differ in protein stability. Importantly, Thr92Ala reduced D2-mediated thyroxine to T3 conversion.

Conclusions: Thyroidectomized patients carrying Thr92Ala are at increased risk of reduced intracellular and serum T3 concentrations that are not adequately compensated for by LT4, thus providing evidence in favor of customized treatment of hypothyroidism in athyreotic patients.
(*J Clin Endocrinol Metab* 102: 1623–1630, 2017)

Although levothyroxine (LT4) replacement is the standard of care for hypothyroid patients, a substantial proportion of such patients treated with it complain of symptoms of hypothyroidism (memory loss, weight gain, fatigue, depression, and reduced quality of life) despite normal values of thyroid-stimulating hormone (TSH) (1–3). In athyreotic patients, both circulating and intracellular triiodothyronine (T3) levels strictly depend on deiodinase-mediated thyroxine (T4) to T3 conversion (4). However, in approximately 20% of patients, LT4 did not ensure

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA
Received 6 July 2016. Accepted 30 January 2017.
First Published Online 2 February 2017

Abbreviations: D1, type 1 deiodinase; D2, type 2 deiodinase; D3, type 3 deiodinase; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; FT3, free triiodothyronine; FT4, free thyroxine; KLF, Kruppel-like factor; K_m, Michaelis constant; LT4, levothyroxine; MuSC, muscle stem cell; PCR, polymerase chain reaction; rT3, reverse T3; T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TSH, thyroid-stimulating hormone.

physiological T3 levels without suppressing TSH (5), although other studies were controversial on this aspect (6,7). LT4 replacement in thyroidectomized animals did not restore euthyroidism in all body tissues, whereas combined T4+T3 therapy did (8). In hypothyroid patients, T4+T3 treatment had no advantage over standard LT4 therapy (9). Indeed, international guidelines recommend only LT4 for hypothyroidism treatment (1, 3). However, LT4 + LT-3 combination therapy might be considered for hypothyroid patients who have persistent complaints despite serum TSH values within the reference range (3).

Conversion of T4 to T3 is normally mediated by type 1 (D1) and type 2 deiodinases (D2). Type 3 deiodinase (D3) inactivates T3 and T4 by converting them into reverse T3 (rT3). D2 has a higher catalytic efficiency than D1, and its K_m for T4 is ~1000 times lower than that of D1. Therefore, in euthyroid humans, D2 accounts for ~70% of circulating T3, whereas only ~15% derives from D1-mediated T4 activation (10).

Human D2 is encoded by the *DIO2* gene. A single nucleotide polymorphism, namely Thr92Ala (rs225014), has been reported in the general population, and the prevalence of the $DIO2^{Ala/Ala}$ homozygous variant ranges between 12.9% and 14.9% (11). Importantly, the effects induced by this polymorphism on D2 enzymatic activity are unclear (12–14). Although clinical studies suggest that the D2-Ala variant may impair D2 enzyme activity (15–18), a clear association between Thr92Ala and reduced tissue T3 levels has not been established.

The aim of this study was to assess the clinical and biochemical significance of the Thr92Ala mutation and other *DIO2* polymorphisms in athyreotic patients.

Patients and Methods

Patient selection

Patients submitted to total thyroidectomy were candidates for this study. Inclusion criteria were thyroid profile data obtained within 10 months of surgery and a postsurgery thyroid profile obtained at least 6 months after achievement of a stable thyroid hormone status on LT4 therapy. In addition, presurgery and postsurgery serum TSH levels should not differ by more than ±0.5 mIU/L. We excluded patients with an abnormal thyroid profile (hypo- or hyperthyroidism) before surgery, patients receiving drugs that could interfere with thyroid function, and patients affected by malabsorption-related conditions. Based on these criteria, we recruited 140 patients for the study (72.1% females) with a mean age of 54.4 ± 14.9 years (range, 18 to 84 years) and with uninodular (73/140, 37.9%) or multinodular goiter (67/140; 62.1%). At final histology, 17/140 (12%) patients had a benign goiter and 123/140 (88%) had differentiated thyroid carcinoma. Immediately after surgery, patients were treated with LT4 to obtain comparable presurgical TSH levels, with a mean dose of 114.9 μg/d and a mean dose/kg of 1.54 μg of LT4. Fasting blood samples were collected at 8 AM to 9 AM before patients assumed the LT4 tablet, and all determinations were performed with a chemiluminescent immunometric assay (Access Immunoassay Systems 2006, Beckman Coulter, Milan, Italy). Normal ranges in our laboratory were 2.5 to 4.1 pg/mL for free T3 (FT3), 5.8 to 16.4 pg/mL for free thyroxine (FT4), and 0.4 to 4.0 mIU/L for TSH. In our laboratory, the interassay variation of the FT3 assay was 8%, which corresponds to ±0.26 pg/mL. Thus, we arbitrarily selected a change of at least 0.5 pg/mL as a significant variation between pre- and postsurgical FT3 value.

DIO2 gene analysis

For gene analysis, 102/140 (72.8%) patients agreed to donate a blood sample for genetic testing. Each patient provided written informed consent to the study. Genomic DNA was extracted with the QIAamp DNA Micro Kit (Qiagen, Milan, Italy) according to the kit's instructions. DNA concentration was assessed with a fluorometer (GloMax Multi Jr, Promega, Milan, Italy). Primers were designed to cover all *DIO2* exons, the intron/exon junctions, and the 5′-UTR region for the analysis of the −258 G/A (rs12885300) polymorphism using Primer 3 (version 0.4.0). Annealing temperature was 60°C and 51°C (35 cycles), respectively, for exons and for the 5′-UTR ($MgCl_2$ concentration ranged from 1.5 to 2.5 mM). In the case of exons, polymerase chain reaction (PCR) products were analyzed with denaturing high-performance liquid chromatography at specific temperatures to verify the presence/absence of mutations/polymorphisms. Positive samples were subjected to direct sequencing (Genechron, Rome, Italy). For −258 G/A, PCR products were digested with the CviKI-1 restriction enzyme (New England Biolabs, Milan, Italy) at 37°C overnight. Fragments were run in a 3% agarose gel stained with ethidium bromide. Genotype determination on the gel was: wild-type subjects showed three fragments at 117, 24, and 4 bp; heterozygous had four fragments at 145, 117, 24, and 4 bp; and mutant homozygous showed a single 145-bp-long fragment. The restriction fragment length polymorphism genotyping method was verified by a 100% concordance rate after random sequencing 15% of the sample.

Cell cultures and transfections

Muscle stem cells (MuSCs) were prepared for fluorescence-activated cell sorting (FACS) analysis using a FACSARIA cell sorter (BD Biosciences, San Jose, CA) and cultured in 1:1 Dulbecco's modified Eagle medium (Gibco Life Technologies, Paisley, UK) and MCDB-201 medium (Sigma-Aldrich, Saint Louis, MO) containing 20% fetal bovine serum (Gibco) and insulin transferrin selenium (Gibco). Cells were plated on matrigel (BD Biosciences) and kept in an incubator (37.0°C, 6.5% CO_2, 3% O_2). Thyrotrophs were isolated from the pituitary gland of D2KO mice according to the protocol reported by Luongo *et al.* (19). Transient transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Cells were then cultured in Dulbecco's modified Eagle medium with an addition of 10% fetal bovine serum.

Plasmids and reagents

T3-T4 and rT3 were purchased from Sigma-Aldrich and used at a 30-nM working concentration. Cycloheximide was from Sigma-Aldrich and used at a 10-μM concentration.

Western blot analysis

Total protein extracts from cells and tissues were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). The membrane was then blocked with 5% nonfat dry milk in phosphate buffered saline, probed with anti-Flag antibody (1:1000, M2-Sigma) or PARP antibody (1:500, Cell Signaling, Danvers, MA) for 2 hours, washed and incubated with horseradish peroxidase–conjugated donkey antirabbit or mouse immunoglobulin G secondary antibody (1:3000), and detected by chemiluminescence (Millipore). After extensive washing, the membrane was incubated with antitubulin-specific antibody (1:10,000; sc-8035, Santa Cruz, Dallas, TX) as loading control. All Western blots were run in triplicate.

Immunofluorescence

For immunofluorescent staining, cells were fixed with 4% formaldehyde and permeabilized in 0.1% Triton X-100, then blocked with 0.5% goat serum and incubated with primary antibody. AlexaFluor 595- or 488-conjugated secondary antibody was used. Immunofluorescence labeling was evaluated with a Zeiss 510 confocal laser scanner microscope and with an IX51 Olympus microscope and the Cell*F software. Images were assembled using Adobe Photoshop.

Statistics

For epidemiological data (presented as mean ± standard deviation) and *DIO2* polymorphism analysis, we used GraphPad Prism 4. The paired *t* test was used to analyze normally distributed data, and the Wilcoxon and the Mann-Whitney tests were used to analyze not normally distributed data. We used contingency tables to evaluate substantial differences in data frequency. One-way analysis of variance with Dunnett *post hoc* test was used for multiple comparisons. For basic experiments, differences between samples were assessed with the Student two-tailed *t* test for independent samples. Errors are reported as standard error of the mean throughout. Differences were considered significant when P was <0.05 ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Results

Comparison of presurgical and postsurgical thyroid hormone levels

As shown in Fig. 1(a), there was no significant difference between presurgery and postsurgery serum TSH levels in any subject (by Wilcoxon test for paired data), which is consistent with the inclusion criteria and the aim of the study. Despite similar TSH levels, mean FT4 levels were slightly but significantly higher ($P < 0.001$) after surgery (10.8 ± 2.2 pg/mL) than before surgery (9.8 ± 2.60 pg/mL). On the contrary, the mean postsurgical serum FT3 level was significantly ($P < 0.001$) lower than the presurgical FT3 level (3.0 ± 0.35 pg/mL vs 3.3 ± 0.43 pg/mL). Consequently, a significantly lower FT3/FT4 ratio was observed in the postsurgical evaluation (0.28 ± 0.06 vs 0.36 ± 0.09; $P < 0.0001$). According to the cutoff defined in the "Patients and Methods" section, patients were classified as having "reduced FT3" when postsurgical FT3 levels were at least 0.5 pg/mL lower than presurgical FT3 values. As shown in Fig. 1(b), postsurgery FT3 levels were lower in 48/140 patients ("reduced FT3," 34.3%), and "unchanged" in the remaining 92 patients (FT3, 65.7%). Notably, sex, age, TSH levels, FT4 levels, the FT4/FT3 ratio, LT4 dose/day, and LT4 dose/kg did not differ between patients, with a lower FT3 level after surgery ("reduced FT3") and those in whom FT3 was unchanged ("unchanged FT3") (Table 1).

Correlation between the DIO2 Thr92Ala polymorphism and serum FT3 levels

To investigate whether the lower postsurgical FT3 level observed in a subset of patients was correlated with deiodinase gene polymorphisms, we analyzed the entire coding region of the *DIO2* gene in 102 samples (72.8% of enrolled patients) and the 5′-UTR region and identified the Thr92Ala polymorphism in 63.7% of subjects: 37/102 (36.3%) patients were homozygous wild-type (Thr/Thr), 52/102 (51.0%) heterozygous (Thr/Ala), and 13/102 (12.7%) mutant homozygous (Ala/Ala). FT4 levels were significantly higher after surgery than before surgery in the Thr/Thr group (11.1 ± 2.4 pg/mL vs 9.5 ± 2.2 pg/mL, $P = 0.001$) and in the Thr/Ala group (10.9 ± 2.0 pg/mL vs 9.9 ± 2.8 pg/mL, $P = 0.02$), and slightly, but not significantly higher in the Ala/Ala group (10.1 ± 1.9 pg/mL vs 9.4 ± 2.6 pg/mL, $P = 0.38$). As shown in Fig. 1(c), mean postsurgery FT3 levels were significantly lower ($P < 0.0001$ in the Thr/Ala group; $P = 0.01$ in the Ala/Ala group) in patients carrying the mutated allele(s) than in wild-type patients, in whom, moreover, FT3 postsurgical levels were similar to presurgery levels ($P = 0.097$).

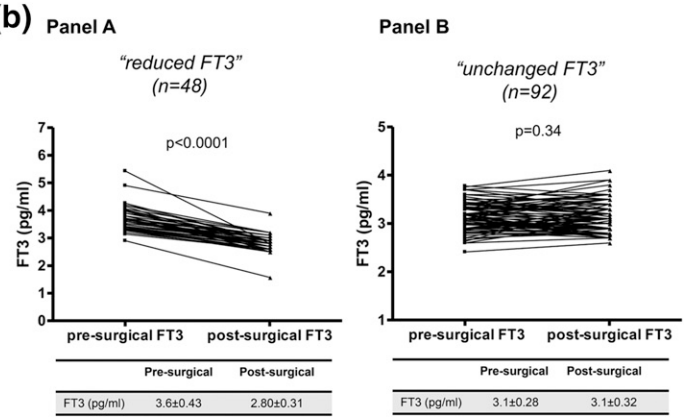
To verify these results, we compared pre- and postsurgical changes in FT3 levels among the three genotype groups. FT3 changes (expressed as the difference between pre- and postsurgical FT3 levels) were significantly higher in the Thr/Ala group ($P < 0.05$; 95% confidence interval, 0.06038 to 0.5459) and in the Ala/Ala group ($P < 0.05$; 95% confidence interval, 0.004411 to 0.7323) than in the Thr/Thr group [Fig. 1(d)]. On the contrary, TSH, FT4, and T3/T4 ratio changes did not differ among the three genotype groups. We also characterized our patients for the −258 G/A polymorphism. We found −258 G/A in 102 (45.8%) patients: 56/102 (54.9%) patients were homozygous wild-type, 35/102 (34.3%) heterozygous, and 11/102 (10.8%) mutant homozygous. This polymorphism was not correlated with FT3 changes even in a subgroup of patients who were wild-type for the Thr92Ala and who were thus not influenced by this variant (data not shown).

(a)

	TSH (mUI/L)	FT3 (pg/ml)	FT4 (pg/ml)
Pre-surgical			
Mean±SD	0.93±0.55	3.3±0.43	9.8±2.6
Range	0.10-3.4	2.4-5.4	5.5-17.7
Median	0.88	3.3	9.3
Post-surgical			
Mean±SD	0.91±0.59	3.0±0.35	10.8±2.2
Range	0.01-3.6	1.6-4.1	5.9-18.3
Median	0.89	2.9	10.7
p pre *versus* post	0.27	<0.0001	<0.0001

NA: not applicable

(b)



(c)

	Pre-surgical FT3 (pg/ml)	Post-surgical FT3 (pg/ml)	p
WT			
Mean±SD	3.2±0.35	3.1±0.36	
Range	2.4-3.9	2.6-4.1	0.097
Median	3.2	3.1	
Thr/Ala			
Mean±SD	3.4±0.52	3.0±0.28	
Range	2.7-5.4	2.5-3.9	<0.0001
Median	3.3	2.9	
Ala/Ala			
Mean±SD	3.4±0.37	2.9±0.34	
Range	2.9-4.2	2.5-3.6	0.01
Median	3.4	2.9	

(d)

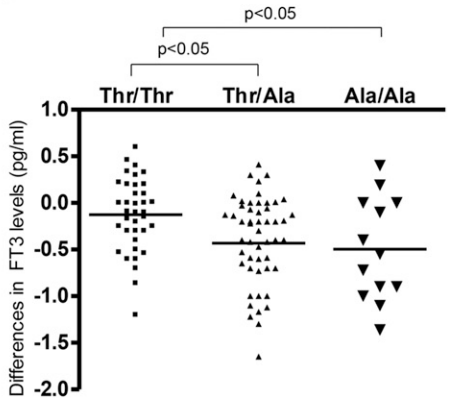


Figure 1. Thyroid hormone levels before and after surgery and correlation with *DIO2* gene polymorphisms. Presurgical and postsurgical thyroid hormone parameters (a). Presurgical and postsurgical FT3 levels in the "reduced FT3" group (b, panel A) and in the "unchanged FT3" (b, panel B) group. Presurgical and postsurgical FT3 levels in relation to the *DIO2* genotype (c). Differences between presurgical and postsurgical serum FT3 levels in the three genotype groups (d). SD, standard deviation; WT, wild-type.

Endogenous D2 protein expressed at low but functional levels in proliferating myoblasts in which both the wild-type and mutant enzymes are dynamically located in the ER

To determine whether D2-Ala modified relevant enzymatic parameters, we first investigated the localization of the wild-type protein in its physiological context. *In vivo* detection of endogenous D2 and identification of its subcellular localization are not possible given the lack of functional antibodies. To overcome this problem, we generated a knock-in 3xFlag-D2 mouse carrying three repetitions of a Flag epitope at the NH2-terminus of the otherwise wild-type D2 enzyme [Supplemental Fig. 1(A) and 1(B)]. Western blotting analysis confirmed the proper expression of the properly functional (data not shown) 3xFlag-D2 protein in the expected D2-expressing tissues [Supplemental Fig. 1(C) and 1(D)].

We used MuSCs as a model to address the intracellular localization of the D2 protein (20). Activated MuSCs isolated by FACS from the 3xFlag-D2 mouse were evaluated for D2 expression by coimmunofluorescence analysis. Proliferating cells expressed low levels of D2, mainly in the cytoplasm [Fig. 2(a)]. D2 partially colocalized with the endoplasmic reticulum (ER) marker calreticulin in early differentiating MuSCs [Fig. 2(b)]. Interestingly, D2 also localized in cytoplasmic vesicles other than the ER (as shown by the absence of calreticulin staining). As D2 expression increased during differentiation, it shifted to a perinuclear space [Fig. 2(b)]. This pattern is identical to that observed when exogenous D2 was transfected in FACS-isolated myoblasts from D2KO mice [Fig. 3(a)].

To determine whether the mutant isoform has the same subcellular localization as the wild-type D2, we transfected MuSCs from D2 knockout mice with D2-WT or D2-Ala plasmid and evaluated its cellular localization. Immunofluorescence staining showed that both transfected isoforms localized to the cytoplasm, mainly to the ER [Fig. 3(a)]. Cell fractionation confirmed that D2 was predominantly retained in microsomes [Fig. 3(b)]. In conclusion, *in vivo* D2 tracing demonstrated that D2 is dynamically expressed in the ER–perinuclear space during cell differentiation and that the D2 expression profiles of the wild-type and mutant D2-Ala isoforms are identical.

Table 1. Clinical, Demographical, and Biochemical Data in "Reduced FT3" and "Unchanged FT3" Patients

	Reduced FT3 (n = 48)	Unchanged FT3 (n = 92)	*P* Value
Postsurgical FT3 (pg/mL; normal range, 2.5–3.9)			<0.0001
Mean ± SD	2.80 ± 0.31	3.1 ± 0.32	
Range	1.57–3.9	2.6–4.1	
Median	2.8	3.1	
Postsurgical FT4 (pg/mL; normal range, 5.8–16.4)			0.17
Mean ± SD	10.53 ± 2.25	11.0 ± 2.1	
Range	5.0–16.9	7.1–18.1	
Median	10.0	10.9	
Postsurgical TSH (mIU/L; normal range, 0.4–4.0)			0.12
Mean ± SD	0.98 ± 0.53	0.87 ± 0.62	
Range	0.13–2.23	0.01–3.62	
Median	0.98	0.74	
Postsurgical FT3/FT4			0.15
Mean ± SD	0.28 ± 0.07	0.29 ± 0.06	
Range	0.14–0.50	0.17–0.4	
Median	0.27	0.29	
Sex			0.55
Males (n, %)	15 (38.4%)	24 (61.6%)	
Females (n, %)	33 (32.6%)	68 (67.4%)	
Age (y)			0.67
Mean ± SD	55.1 ± 16.0	54.0 ± 14.4	
Range	24–84	18–84	
Median	55.5	57	
LT4 daily dose (μg)			0.27
Mean ± SD	111.3 ± 30.6	116.8 ± 25.6	
Range	15–175	62–175	
Median	112.5	112.5	
Pro-kg LT4 dose (μg)			0.17
Mean ± SD	1.48 ± 0.36	1.56 ± 0.33	
Range	0.2–2.36	0.9–2.8	
Median	1.5	1.5	

Abbreviation: SD, standard deviation.

Because Thr92Ala lies in a specific 18-amino-acid large loop critical for D2 recognition by the WSB-1 subunit of E3 ubiquitin ligase (21), we compared the protein stability of the wild-type and mutant isoforms. We evaluated the clearance rate of D2 after administration of rT3, which accelerates D2 proteolytic degradation. The D2-Ala protein was even more stable under these conditions and had a longer half-life than wild-type D2 [Supplemental Fig. 2(A) and 2(B)]. Similarly, when cycloheximide was used to inhibit protein biosynthesis, the decrease of D2-Ala clearance was lower than that of D2-WT [Supplemental Fig. 2(C) and 2(D)].

The D2-Ala mutant is less active *in vivo* than the wild-type D2 enzyme

Proliferating myoblasts express elevated D3 levels (22). The finding of active D2 in proliferating myoblasts was somewhat unexpected given the abundant D3 expression in these cells. Indeed, temporary thyroid hormone (TH) excess by D3-depletion has been reported to lead to apoptosis of activated proliferating MuSCs (23). To assess whether forced amounts of D2 might disrupt the balance of intracellular TH and cause apoptosis, we transfected proliferating myoblasts with D2. In these cells, D2 caused dose-dependent apoptosis as demonstrated by PARP cleavage, thus confirming that a D2-dependent increase in intracellular T3 causes cell death during proliferation [Supplemental Fig. 2(E)].

The D2-dependent proapoptotic effect in proliferating myoblasts suggested that this cellular model might be a biological sensor with which to quantitatively assess D2-mediated T4 to T3 conversion. Consequently, we asked whether also the D2-Ala mutant isoform is equally able to produce T3 and therefore induce apoptosis. To this aim, we transfected myoblasts from *Dio2-null* mice with identical amounts of D2-WT or D2-Ala. Strikingly, the D2-Ala mutant caused less apoptosis than the wild-type, which suggests a lower intracellular T4 to T3 conversion by D2-Ala [Fig. 3(c)]. The effect of D2-Ala on apoptosis was dose-dependent, because higher amounts of transfected plasmid induced a response similar to that obtained with D2-WT [Fig. 3(d)]. In conclusion, these data demonstrate that, despite a normal

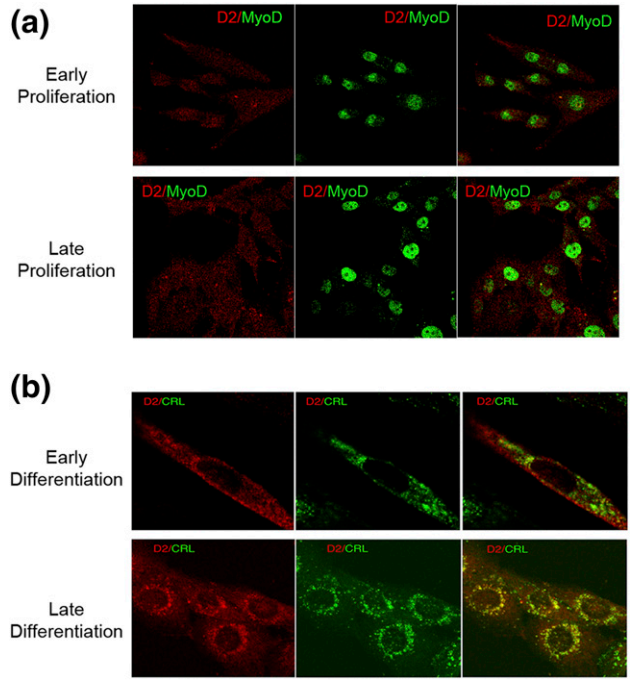


Figure 2. D2 localizes in the endothelial reticulum and cytoplasmic vesicles in proliferating and differentiated MuSCs. D2 localization was evaluated by immunofluorescence microscopy in FACS-isolated MuSCs cultured in proliferative and differentiative conditions at different time points. Early proliferative cells were fixed at 12 hours after plating, late proliferative cells were fixed 24 hours after plating; differentiation was induced 48 hours following plating by replacing growing medium with differentiation medium and collected at 12 (early) and 48 hours following medium replacement. Proliferating cells were costained with Flag/MyoD antibodies and differentiated cells with Flag/calreticulin antibodies as indicated.

subcellular localization and an even slightly higher protein stability, the D2-Ala mutant is less efficient than D2-WT in converting T4 into T3 in muscle cells *in vivo*.

To determine the catalytic efficiency of the D2-Ala protein in a different biological setting, we transfected D2-Ala in primary cultures of pituitary cells from *Dio2-null* mice and determined its efficiency in blocking TSH synthesis. The response to T4-induced TSH suppression was significantly ($P < 0.05$) lower in thyrotrophs expressing D2-Ala than in D2-WT-treated thyrotrophs [Fig. 3(e)], again demonstrating an impairment in the T4 to T3 conversion by the mutant enzyme.

Discussion

In this study, we provide *in vivo* biological evidence that the D2-Ala enzyme is less enzymatically efficient and reduces T3 production in two relevant D2-dependent tissues (*i.e.*, skeletal muscle and pituitary) as well as in the serum of athyreotic patients. In a previous study, D2 activity was reduced in muscle samples from patients homozygous for the Ala allele (13); however, that finding may have been related to an artifact intrinsic to the D2 activity *in vitro* assay (4, 24). To overcome the limitations of the D2 *in vitro* assay, we used an *in vivo* system in intact cells that more closely resemble the *in vivo* situation and that enabled us to identify a functional limitation resulting from the D2-Ala mutation. Using this approach, we demonstrate that, notwithstanding a normal subcellular localization and even a slightly increased protein stability, the D2 Ala mutant is less efficient *in vivo* in converting T4 into T3 in muscle cells.

In athyreotic patients in therapy with LT4 substitution, both circulating and intracellular T3 levels strictly depend on the deiodinase-mediated T4 to T3 conversion (4). However, in approximately 20% of athyreotic patients, LT4 did not ensure physiological T3 levels unless suppressing TSH (5), although other studies were controversial on this aspect (5, 6). In view of these controversies, we compared thyroid hormone levels (TSH, FT3, and FT4) before and after total thyroidectomy on LT4-replacement therapy in patients who had similar TSH levels both before and after surgery. We found that postoperative FT3 levels were significantly reduced in a subgroup of patients (34.3%) submitted to total thyroidectomy and undergoing LT4 therapy even though their TSH levels were in the normal range and similar to those observed in the presurgical evaluation. Although clinical studies suggest that enzymatic activity may be impaired in patients carrying the D2-Ala polymorphism (15–18), a clear association between Thr92Ala and reduced tissue T3 levels has not been established. Previous studies of thyroid hormone levels in patients genotyped for DIO2 Thr92Ala, and their respective LT4 intake, yielded conflicting results (25), mainly from the lack of presurgery hormonal values and to the heterogeneity of study populations.

To investigate whether the lower postsurgical FT3 that we observed in a subset of patients was correlated with deiodinase gene mutations, we analyzed the entire coding region and the 5′-UTR of the *DIO2* gene in 102 samples (72.8% of the enrolled patients). We found two polymorphisms: the Thr92Ala and the −258 G/A. Within our experimental design, patients carrying the mutated allele(s) for the Thr92Ala had significantly lower postsurgery FT3 levels compared with wild-type subjects in which FT3 postsurgery levels were similar to presurgical levels. The percentage of reduced postsurgery FT3 levels was directly correlated with the presence and severity of the polymorphism: 58.3% in homozygous (Ala/Ala) patients vs 36.5% in heterozygous (Thr/Ala) subjects. The −258 G/A variant did not affect FT3 levels even in a subgroup of patients wild-type for Thr92Ala.

To rule out that these low FT3 values could be correlated to lower FT4 levels, we evaluated presurgical and postsurgical FT4 levels in relation to genotype. FT4 levels were

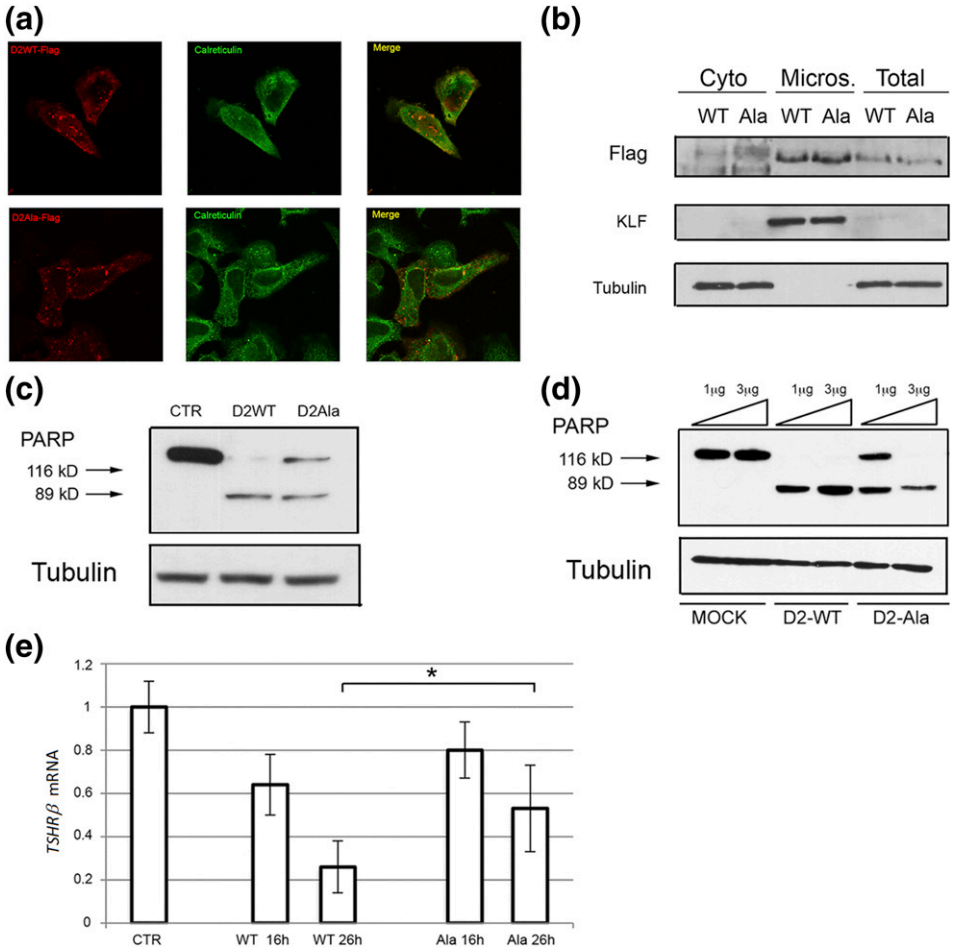


Figure 3. Wild-type D2 and D2-Ala proteins share the same localization, but differ in activity. (a) Immunofluorescence staining for D2-WT or D2-Ala (red) and calreticulin (green) in transiently transfected MuSCs from D2KO mice. (b) D2wt- and D2-Ala–expressing plasmids were transiently transfected in MuSCs from D2KO mice. Twenty-four hours later, cells were fractionated into cytosolic (cyto) and ER-containing microsomal (micros) fractions. The presence of D2 in the two compartments was determined by Western blot analysis with anti-Flag antibody. KDEL (as ER marker) and tubulin were used as loading controls. KLF, Kruppel-like factor. (c) Western blot analysis of PARP cleavage in proliferating MuSCs from D2KO mice 48 hours after transfection with D2-WT and D2-Ala, and after 24 hours incubation with 30 nM T4. Tubulin served as loading control. (d) Dose-response effect of D2-Ala on apoptosis of proliferating MuSCs. MuSCs from D2KO mice were transiently transfected with 1 µg and 3 µg of D2-WT and D2-Ala plasmid, respectively. Twenty-four hours later, cells were treated with 30 nM T4. Total proteins were harvested 48 hours later for Western blot analysis of PARP cleavage. Tubulin served as loading control. (e) Time course reverse transcription PCR analysis of β-TSH subunit expression in thyrotrophs from D2KO mice, cultured in charcoal-stripped serum, transiently transfected with D2-WT and D2-Ala, 16 and 26 hours after 30 nM T4 incubation, respectively. Nontreated thyrotrophs served as control (CTR).

significantly higher postsurgery than presurgery in all three subgroups (data not shown). In addition, postoperative FT4 levels did not differ among the three genotype groups.

These findings support our hypothesize that lower postsurgical levels of FT3 in Thr/Ala and Ala/Ala subjects could be related to the presence of the D2 polymorphism rather than to lower FT4 levels in Ala/Ala and Thr/Ala patients vs Thr/ Thr patients.

The strength of our study is that it was conducted in patients that had similar presurgical and postsurgery TSH levels, thus enabling us to evaluate putative changes in circulating TH levels with the same feedback set point. A limitation of our study is that, being unable to perform a replicative study of the deiodinase gene mutations in an independent cohort, we cannot exclude the presence of false-positive results.

In conclusion, we demonstrate that a lower plasmatic T3 level is associated with the X/Ala D2 genotype, which suggests that a segment of these patients do not respond optimally to standard LT4 therapy. It is reasonable to speculate that in the "FT3-reduced" group of patients, restoration of presurgery FT3 values would be obtained by increasing the LT4 dose at the expense of a higher plasmatic LT4 level and a lower TSH level (in the range of subclinical hypothyroidism) or by adding T3. In this context, our study might support the use of combined T4+T3 therapy in the subgroup with low serum T3 that carries D2-Ala mutants.

Acknowledgments

We thank Jean Ann Gilder (Scientific Communication srl., Naples, Italy) for editing the manuscript.

Address all correspondence and requests for reprints to: Domenico Salvatore, MD, PhD, Department of Clinical Medicine and Surgery, University of Naples "Federico II," Via S. Pansini 5, 80131 Naples, Italy. E-mail: domenico.salvatore@unina.it.

This work was supported in part by grants from the Ministero Italiano dell'Università e Ricerca (Grant 2012Z3F7HE).

Author contributions: M.C.G. and M.D. designed the research studies, conducted experiments, and wrote the manuscript. F.M. and R.A. designed the research studies and acquired data. S.C., T.P., and C.M. conducted experiments. C.G. acquired and analyzed the immunofluorescence studies. F.P. and D.S. designed the study, supervised the experiments, analyzed the results, and wrote the paper. All authors discussed the results and gave input on the manuscript.

Disclosure Summary: The authors have nothing to disclose.

References

1. Jonklaas J, Bianco AC, Bauer AJ, Burman KD, Cappola AR, Celi FS, Cooper DS, Kim BW, Peeters RP, Rosenthal MS, Sawka AM; American Thyroid Association Task Force on Thyroid Hormone Replacement. Guidelines for the treatment of hypothyroidism: prepared by the american thyroid association task force on thyroid hormone replacement. *Thyroid*. 2014;**24**(12):1670–1751.
2. Wekking EM, Appelhof BC, Fliers E, Schene AH, Huyser J, Tijssen JG, Wiersinga WM. Cognitive functioning and well-being in euthyroid patients on thyroxine replacement therapy for primary hypothyroidism. *Eur J Endocrinol*. 2005;**153**(6):747–753.
3. Wiersinga WM, Duntas L, Fadeyev V, Nygaard B, Vanderpump MP. 2012 ETA Guidelines: the use of L-T4 + L-T3 in the treatment of hypothyroidism. *Eur Thyroid J*. 2012;**1**(2):55–71.
4. Acosta BM, Bianco AC. New insights into thyroid hormone replacement therapy. *F1000 Med Rep*. 2010;**2**:2.
5. Gullo D, Latina A, Frasca F, Le Moli R, Pellegriti G, Vigneri R. Levothyroxine monotherapy cannot guarantee euthyroidism in all athyreotic patients. *PLoS One*. 2011;**6**(8):e22552.
6. Jonklaas J, Davidson B, Bhagat S, Soldin SJ. Triiodothyronine levels in athyreotic individuals during levothyroxine therapy. *JAMA*. 2008;**299**(7):769–777.
7. Ito M, Miyauchi A, Morita S, Kudo T, Nishihara E, Kihara M, Takamura Y, Ito Y, Kobayashi K, Miya A, Kubota S, Amino N. TSH-suppressive doses of levothyroxine are required to achieve preoperative native serum triiodothyronine levels in patients who have undergone total thyroidectomy. *Eur J Endocrinol*. 2012;**167**(3):373–378.
8. Escobar-Morreale HF, Obregón MJ, Escobar del Rey F, Morreale de Escobar G. Replacement therapy for hypothyroidism with thyroxine alone does not ensure euthyroidism in all tissues, as studied in thyroidectomized rats. *J Clin Invest*. 1995;**96**(6):2828–2838.
9. Grozinsky-Glasberg S, Fraser A, Nahshoni E, Weizman A, Leibovici L. Thyroxine-triiodothyronine combination therapy versus thyroxine monotherapy for clinical hypothyroidism: meta-analysis of randomized controlled trials. *J Clin Endocrinol Metab*. 2006;**91**(7):2592–2599.
10. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev*. 2002;**23**(1):38–89.
11. Grarup N, Andersen MK, Andreasen CH, Albrechtsen A, Borch-Johnsen K, Jørgensen T, Auwerx J, Schmitz O, Hansen T, Pedersen O. Studies of the common DIO2 Thr92Ala polymorphism and metabolic phenotypes in 7342 Danish white subjects. *J Clin Endocrinol Metab*. 2007;**92**(1):363–366.
12. Peeters RP, van Toor H, Klootwijk W, de Rijke YB, Kuiper GG, Uitterlinden AG, Visser TJ. Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects. *J Clin Endocrinol Metab*. 2003;**88**(6):2880–2888.
13. Canani LH, Capp C, Dora JM, Meyer EL, Wagner MS, Harney JW, Larsen PR, Gross JL, Bianco AC, Maia AL. The type 2 deiodinase A/G (Thr92Ala) polymorphism is associated with decreased enzyme velocity and increased insulin resistance in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2005;**90**(6):3472–3478.
14. McAninch EA, Jo S, Preite NZ, Farkas E, Mohácsik P, Fekete C, Egri P, Gereben B, Li Y, Deng Y, Patti ME, Zevenbergen C, Peeters RP, Mash DC, Bianco AC. Prevalent polymorphism in thyroid hormone-activating enzyme leaves a genetic fingerprint that underlies associated clinical syndromes. *J Clin Endocrinol Metab*. 2015;**100**(3):920–933.
15. Dora JM, Machado WE, Rheinheimer J, Crispim D, Maia AL. Association of the type 2 deiodinase Thr92Ala polymorphism with type 2 diabetes: case-control study and meta-analysis. *Eur J Endocrinol*. 2010;**163**(3):427–434.
16. Torlontano M, Durante C, Torrente I, Crocetti U, Augello G, Ronga G, Montesano T, Travascio L, Verrienti A, Bruno R, Santini S, D'Arcangelo P, Dallapiccola B, Filetti S, Trischitta V. Type 2 deiodinase polymorphism (threonine 92 alanine) predicts L-thyroxine dose to achieve target thyrotropin levels in thyroidectomized patients. *J Clin Endocrinol Metab*. 2008;**93**(3):910–913.
17. He B, Li J, Wang G, Ju W, Lu Y, Shi Y, He L, Zhong N. Association of genetic polymorphisms in the type II deiodinase gene with bipolar disorder in a subset of Chinese population. *Prog Neuropsychopharmacol Biol Psychiatry*. 2009;**33**(6):986–990.
18. Butler PW, Smith SM, Linderman JD, Brychta RJ, Alberobello AT, Dubaz OM, Luzon JA, Skarulis MC, Cochran CS, Wesley RA, Pucino F, Celi FS. The Thr92Ala 5′ type 2 deiodinase gene polymorphism is associated with a delayed triiodothyronine secretion in response to the thyrotropin-releasing hormone-stimulation test: a pharmacogenomic study. *Thyroid*. 2010;**20**(12):1407–1412.
19. Luongo C, Martin C, Vella K, Marsili A, Ambrosio R, Dentice M, Harney JW, Salvatore D, Zavacki AM, Larsen PR. The selective loss of the type 2 iodothyronine deiodinase in mouse thyrotrophs increases basal TSH but blunts the thyrotropin response to hypothyroidism. *Endocrinology*. 2015;**156**(2):745–754.
20. Dentice M, Marsili A, Ambrosio R, Guardiola O, Sibilio A, Paik JH, Minchiotti G, DePinho RA, Fenzi G, Larsen PR, Salvatore D. The FoxO3/type 2 deiodinase pathway is required for normal mouse myogenesis and muscle regeneration. *J Clin Invest*. 2010;**120**(11):4021–4030.
21. Dentice M, Bandyopadhyay A, Gereben B, Callebaut I, Christoffolete MA, Kim BW, Nissim S, Mornon JP, Zavacki AM, Zeöld A, Capelo LP, Curcio-Morelli C, Ribeiro R, Harney JW, Tabin CJ, Bianco AC. The Hedgehog-inducible ubiquitin ligase subunit WSB-1 modulates thyroid hormone activation and PTHrP secretion in the developing growth plate. *Nat Cell Biol*. 2005;**7**(7):698–705.
22. Salvatore D, Simonides WS, Dentice M, Zavacki AM, Larsen PR. Thyroid hormones and skeletal muscle—new insights and potential implications. *Nat Rev Endocrinol*. 2014;**10**(4):206–214.
23. Dentice M, Ambrosio R, Damiano V, Sibilio A, Luongo C, Guardiola O, Yennek S, Zordan P, Minchiotti G, Colao A, Marsili A, Brunelli S, Del Vecchio L, Larsen PR, Tajbakhsh S, Salvatore D. Intracellular inactivation of thyroid hormone is a survival mechanism for muscle stem cell proliferation and lineage progression. *Cell Metab*. 2014;**20**(6):1038–1048.
24. Larsen PR. Type 2 iodothyronine deiodinase in human skeletal muscle: new insights into its physiological role and regulation. *J Clin Endocrinol Metab*. 2009;**94**(6):1893–1895.
25. Heemstra KA, Hoftijzer HC, van der Deure WM, Peeters RP, Fliers E, Appelhof BC, Wiersinga WM, Corssmit EP, Visser TJ, Smit JW. Thr92Ala polymorphism in the type 2 deiodinase is not associated with T4 dose in athyroid patients or patients with Hashimoto thyroiditis. *Clin Endocrinol (Oxf)*. 2009;**71**(2):279–283.