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Prevalence of *Dio2*^{T92A} polymorphism and its association with thyroid autoimmunity

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Running Head: *Dio2*^{T92A} polymorphism

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Abstract

The 3,5,3′-L-triiodothyronine (T3) partly derives by the deiodination of the prohormone 3,5,3′,5’-L-tetraiodothyronine (T4) by the type 2 iodothyronine deiodinase (D2). The single-nucleotide polymorphism in the D2 gene at position 92 (*Dio2*^{T92A}), generates an enzyme with a reduced T4 to T3 conversion velocity. Because thyroid hormones can modulate the immune response, we hypothesized a pathophysiological role for *Dio2*^{T92A} polymorphism in autoimmunity. The objective of this study was to investigate the *Dio2*^{T92A} polymorphism in relation to thyroid autoimmunity. We compared the prevalence of *Dio2*^{T92A} polymorphism and serum thyroid hormone levels in healthy subjects and subjects with thyroid autoimmunity (TA). A total of 110 subjects with TA and 106 controls were genotypized for *Dio2*^{T92A} polymorphism. Free T3 (FT3), free T4 (FT4) and thyrotropin (TSH) were measured and compared with the *Dio2*^{T92A} polymorphism. *Dio2*$^{92T/A}$, *Dio2*$^{92A/A}$ and *Dio2*$^{92T/T}$ healthy subjects were 40.9%, 46.4%, and 12.7% respectively. These prevalences were similar to those of some European countries whilst significantly different from that of Brazil. In the two groups of healthy subjects and TA subjects, *Dio2*^{T92A} polymorphism had a similar distribution with non significant differences. Similarly, no significant differences were observed in the serum concentration of FT3, FT4 and TSH between subjects with different *Dio2*^{T92A} polymorphism. The FT4/FT3 and TSH/FT3 ratios were higher in *Dio2*$^{92T/T}$ than in *Dio2*$^{92T/A}$ and *Dio2*$^{92A/A}$ subjects in both TA and healthy groups, but these differences were not significant. In conclusion, the distribution of *Dio2*^{T92A} polymorphism may reflect geographical and ethnic differences, and it is not associated with thyroid autoimmunity.

Key words: type 2 iodothyronine deiodinase, *Dio2*^{T92A} polymorphism, thyroid autoimmunity

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Introduction

The 3,5,3′-L-triiodothyronine (T3) is the biologically active thyroid hormone. T3 is partly produced and released into the serum by the thyroid and partly derives by the deiodination of the prohormone 3,5,3′,5’-L-tetraiodothyronine (T4) (1). Type 2 iodothyronine deiodinase (D2), catalyzes removal of an outer ring 5′-iodine atom from the circulating T4 to generate the active metabolite T3. Thus, D2 in conjunction with thyroid-derived T3, is an important local modulator of thyroid hormone action. D2-mediated T3 production by T4 occurs at the intracellular compartment. Subsequently, T3 exists the cells and enters the plasma compartment, being responsible for 70% of all extrathyroidal T3 production in healthy humans (2). Expression of D2 is regulated in a tissue-specific manner, resulting in varying levels of T3 action in individual tissues, despite relatively constant serum thyroid hormone levels (1). D2 is a protein residing on the endoplasmic reticulum membrane, with a relatively short half-life due to ubiquitination and proteasome degradation (3). D2 expression is regulated on a cell-specific manner by a combination of transcriptional modulation of the *Dio2* gene, post-transcriptional mechanisms regulating *Dio2* mRNA stability, and by post-translational mechanisms such as ubiquitination (1). Several polymorphisms in *Dio2* have been described. The single-nucleotide polymorphism in *Dio2* A92G (D2 Thr92Ala, $Dio2^{T92A}$), generates an enzyme with conserved level of activity. However, the maximal velocity of T4 to T3 conversion results decreased by 3- to 10-fold in thyroid and skeletal muscle of carriers of the $Dio2^{T92A}$. This effect was observed in the absence of differences in D2 mRNA level, suggesting that either the Thr92Ala substitution affects protein translation or stability or a functionally relevant polymorphism occurs in linkage disequilibrium in the $Dio2^{T92A}$ (4). Recent studies have shown that $Dio2^{T92A}$ may have relevant pathophysiological effects. Although discording results have been published, it has been proposed an association of $Dio2^{T92A}$ with an approximately 20% lower glucose disposal rate, with obesity and insulin resistance in subjects with type 2 diabetes mellitus, and with bone mineral density and bone turnover (4-7). It has also been proposed that hypothyroid subjects carrying the $Dio2^{T92A}$ polymorphism in T4 replacement treatment, may experience impaired psychological well-being and would benefit from the T4/T3 combination therapy (8). A large body of evidence indicates that thyroid hormones act as modulators of the immune response. In general, thyroid hormones increases the immune response i.e.: antibody production, cell migration, lymphocyte proliferation, and reactive oxygen species production, whereas it decreases the proinflammatory markers, antioxidant enzymes and their activity (9, 10). Hypothyroidism typically produces the opposite effects on parameters of the immune function; it decreases immune response, antibody production, T cell migration, and lymphocyte proliferation (11, 12). The *Dio2* polymorphism,

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modulating the level of T3 in the lymphoid organs or in the thyroid, could modulate the immune system and thus might affect the tendency to develop thyroid autoimmunity and its course. An hypothesis never investigated so far. Thus, given the putative role of thyroid hormone as modulators of the immune response, and the emerging pathophysiological importance of the *Dio2*^{T92A} polymorphism, the objective of this study was to investigate the *Dio2*^{T92A} polymorphism in relation to thyroid autoimmunity. We compared the prevalence of *Dio2*^{T92A} polymorphism and serum thyroid hormone levels in healthy and subjects with thyroid autoimmunity (TA), seeking a possible correlation between this polymorphism and TA.

Subjects and Methods

Subjects and biochemical measurements

A total of 216 subjects entered in the study following approval from the institutional review board, after giving written consent. Inclusion criteria were female gender, age 25–55 years, absence of thyroid peroxidase antibodies (TPO-Ab) or thyroglobulin antibodies (TG-Ab) (control group) or TPO-Ab titer ≥100 U/ml (at present and ever documented in the past) (thyroid autoimmunity group, TA group), serum TSH values from 0.27–4.0 μIU/ml. Exclusion criteria were male gender, a body mass index < 18.5 or > 28, more than 6 month of amenorrhea, pregnancy, a history of pituitary disease, hyperthyroidism or hypothyroidism at present, or use of any medication. Buccal swab and blood samples were drawn for assessment of *Dio2*^{T92A} polymorphism, TSH, free T_4 (FT_4), free T3 (FT3), TG-Ab and TPO-Ab. Serum concentrations of FT4, FT 3, and TSH were determined by electrochemiluminescent assay using commercially available kits (Roche, Mannheim, Germany). The normal ranges for serum FT4 and FT3 were 7.8-14.2 ng/dl and 2.9-5.8 pg/ml, respectively. The normal range for serum TSH was 0.27-4.0 μIU/ml. TG-Ab and TPO-Ab were determined by a RIA kit (B.R.A.H.M.S. Diagnostica, Berlin, Germany). TPO-Ab <100 U/ml were considered negative.

Dio2^{T92A} polymorphism genotyping

Cotton buccal swabs were stored dry at room temperature up to 3 months before usage. To obtain genomic DNA, buccal swabs were immersed in 400 μl Lysis buffer and resuspended in 10 μL diethylpyrocarbonate (DEPC) water. DNA concentration was quantitated by A 260 absorbance with a BioPhotometer (Eppendorf, Hamburg, Germany). PCR amplification and pyrosequencing analysis PCR were performed with 50–100 ng genomic DNA, forward primer and reverse 5-biotinylated primer (Table 2) at 10M concentration, and 2.5 U Taq Polymerase Recombinant (Fermentas,

Thermo Scientific, Canada). All primers were obtained from Primm (Milan, Italy). All PCR were performed separately in a TC-4000 Thermal Cycler (Bibby Scientific, Milan, Italy), including an initial denaturation of 5 min at 94 C and subsequent denaturation for 20 sec at 94 C, annealing for 20 sec at 56 C, and extension for 30 sec at 72 C. The PCR products were electrophoresed in a 2.5% agarose gel containing ethidium bromide to confirm successful amplification of the PCR products. Preparation of the single-stranded DNA template for pyrosequencing was performed using the PSQ Vacuum Prep Tool (Diatech, Ancona, Italy) according to the manufacturer's instructions. Twenty microliters of biotinylated PCR product were immobilized on streptavidin-coated Sepharose high-performance beads (Diatech), processed to obtain a single-stranded DNA using the PSQ 96 Sample Preparation Kit (Diatech), according to the manufacturer's instructions, and incubated under shaking at room temperature for 10 min in binding buffer. Subsequently, samples were hybridized to 13.5 μM sequencing primers (Table 2) in annealing buffer at 80 C for 2 min in a PSQ96 plate, followed by cooling to room temperature. The sequencing-by-synthesis reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage, Uppsala, Sweden) at room temperature using PyroGold reagents (Diatech). As nucleotides were dispensed, a light signal was generated proportional to the amount of each incorporated nucleotide. These light signals were detected by a charge-coupled device camera and converted to peaks in a sequencing pyrogram that was automatically generated in real time for each sample.

Statistical analysis

Values are presented as percentage or mean +/- standard deviation (s.d.). Comparisons between groups were analyzed by Student's t-test, ANOVA or chi-square tests. All calculations were performed using SPSS 12.0 for windows (SPSS, Inc., Chicago, IL, USA). The level of significance was set at < 0.05.

Results

A total of 216 subjects, 110 with thyroid autoimmunity documented by the presence of serum autoantibodies and 106 controls were genotypized for $Dio2^{T92A}$ polymorphism by pyrosequencing. Sufficient DNA was extracted from all the swabs and sequencing was always successful. $Dio2^{92T/A}$, $Dio2^{92A/A}$ and $Dio2^{92T/T}$ subjects were 41.2%, 44.9%, and 13.9% respectively. In the two groups of healthy subjects and AT subjects, $Dio2^{T92A}$ polymorphism had a similar distribution and non significant differences were observed (Table 3). Similarly, no significant differences were observed in the serum concentration of TSH and FT4 between subjects with different $Dio2^{T92A}$

polymorphism. Serum FT3 in *Dio2*$^{92T/T}$ subjects was slightly lower than in *Dio2*$^{92T/A}$ and *Dio2*$^{92A/A}$ subjects in both groups. However the differences were not significant. Similarly, the FT4/FT3 and TSH/FT3 ratios were higher in *Dio2*$^{92T/T}$ subjects than in *Dio2*$^{92T/A}$ and *Dio2*$^{92A/A}$ subjects in both groups, but these differences were not significant.

Discussion

Recent studies proposed an association of *Dio2*^{T92A} polymorphism with metabolic traits, type 2 diabetes, bone mineral density and Alzheimer disease. Thus, given the emerging physio-pathological importance of D2, the objective of this study was to investigate the *Dio2*^{T92A} polymorphism in relation to thyroid autoimmunity, and circulating thyroid hormones in a large cohort of women from the same geographical area.

The first objective of this study was to investigate whether *Dio2* polymorphism at position 92 is associated with thyroid autoimmunity. The distribution of *Dio2*^{T92A} polymorphism in our Italian cohort of healthy subjects is comparable to that one reported previously in subjects from Denmark and The Netherlands, while the percentage of *Dio2*$^{92A/A}$ subjects (12.7%) was significantly lower than in subjects from Brazil (36.0%) (4, 6, 7). All subjects of our cohort were resident in Campania, thus geographical and ethnic factors could account for the different prevalence observed. No significant association of the *Dio2*^{T92A} polymorphism with thyroid autoimmunity was observed. Our analysis considered subjects with thyroid autoimmunity as documented by the presence of TPO-Ab, and normal level of serum TSH and iodothyronines. Thus, while polymorphism of *Dio2*^{T92A} was not associated with thyroid autoimmunity, its association with Hashimoto's thyroiditis or consequential thyroidal status should be investigated.

The *Dio2*$^{92T/T}$ genotype is associated with a lower D2 enzyme velocity (4). Because extra-thyroidal T4 deiodination by D2 significantly contributes to global T3 production in healthy subjects, we asked whether *Dio2*^{T92A} polymorphism was associated with a different serum level of iodothyronine and TSH/T3 ratio. To analyze a more homogeneous population, we excluded elderly or young subjects, or subjects with a body mass index > 28. This restriction was operated because muscle mass largely expresses D2 and it is age-related, while obesity can influence or can be associated with the level of circulating iodothyronines (4, 5). In both healthy subjects and patients with thyroid autoimmunity, *Dio2*^{T92A} polymorphism was not associated with a variation in TSH or FT4 serum concentration. Serum FT3 level was slightly lower in *Dio2*$^{92T/T}$ subjects than in the subjects

with the two other polymorphisms, a difference that affected the TSH/FT3 and FT4/FT3 ratios. However these differences were not significant. The lack of a different TSH/FT3 ratio as a consequence of *Dio2*^{T92A} polymorphism suggests the possibility that an increased T3 of thyroidal origin counteracts the reduced peripheral T4 to T3 conversion in *Dio2*$^{92A/A}$ subjects. Whilst this may reflect a physiological situation in healthy individuals, a less efficient peripheral T4 to T3 conversion could have metabolic and clinical relevance in hypothyroid subjects treated with substitutive T4 therapy.

A fair proportion (about 13-15%) of hypothyroid patients treated with oral administration of T4 alone, deplores the persistence of symptoms such as feeling tired and lethargic, putting on weight, clumsiness, fatigue, reduced exercise tolerance, muscle weakness (13, 14). It has been postulated that in these patients, deiodination of T4 is less efficient than in others and, although the serum T3 is within the normal range, the final concentration of T3 is low in some tissues. Different studies compared the effects of T4 alone with T4 plus T3 treatment in hypothyroid patients, showing controversial results. Bunevicius et al. reported that the T4/T3 combined replacement therapy of hypothyroidism resulted in improved scores in mood scales and neurocognitive tests in patients under combined treatment while no changes were found in cardiovascular function, body weight and composition and endocrine functions (15, 16). The remarkable finding of advantageous effects of T4/T3 combined therapy on well-being and neurocognitive functions was further studied in other clinical trials, showing inconstant differences between T4 monotherapy and combined therapy (17-19). Recently, Panicker et al. found that hypothyroid patients sharing *Dio2*$^{92A/A}$ genotype had a poorer psychological well-being under T4 monotherapy but they shared a greater psychological improvement under combined T4/T3 therapy (8). In conclusion, the distribution of *Dio2*^{T92A} polymorphism may reflect geographical and ethnic differences, and it is not associated with thyroid autoimmunity. Its association with Hashimoto's thyroiditis, its clinical course and substitutive treatment should be investigated.

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Tables

Table 1. Characteristics of the subjects.

	Healthy subjects n= 106	TA patients n= 110	*P*
Age, yr[a]	40.3 +/- 9.7	41.7 +/- 9.6	0.925
TSH (μIU/ml)[a]	1.98 +/- 0.64	1.93 +/- 0.69	0.852
FT4 (ng/ml)[a]	10.68 +/- 1.73	9.45 +/- 1.58	0.345
FT3 (ng/ml)[a]	3.34 +/- 0.55	3.15 +/- 0.76	0.154
TPO-Ab[b]	<100	1254, (100-9300)	0.197

[a]Mean, +/- s.d.; [b]median, (range). Analysis was by Student's t-test. Reference values: TSH, 0.27–4.0; FT4, 7.8-14.2; FT3, 2.9-5.8; TPO-Ab, <100

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Table 2. PCR primers and sequencing primers for *Dio2*.

PCR primers	Sequencing primers
Forward: 5'-ATTCCAGTGTGGTGCATGTC -3' Reverse: 5'-biotin-GCTCGTGAAAGGAGGTCAAG -3'	5'-TGGTGCATGTCTCCA-3'

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Table 3. Prevalence of *Dio2* polymorphism in healthy subjects and TA patients. Percentages.

	Healthy subjects	TA patients	*P*
$Dio2^{92T/T}$	40.9	38.7	0.829
$Dio2^{92T/A}$	46.4	47.2	1.000
$Dio2^{92A/A}$	12.7	14.2	0.710

Analysis was by chi-square of independence test.

Table 4. Correlation of serum TSH and iodothyronine levels with *Dio2* polymorphism in healthy subjects and AT patients.

	Healthy subjects			TA patients		
	Dio2$^{92T/T}$	*Dio2*$^{92T/A}$	*Dio2*$^{92A/A}$	*Dio2*$^{92T/T}$	*Dio2*$^{92T/A}$	*Dio2*$^{92A/A}$
Age, mean yr	46.3	49.1	44.5	44.3	45.4	48.3
TSH (μIU/ml) [a]	1.99 +/- 0.64	1.97 +/- 0.75	1.96+/- 0.76	1.95 +/- 0.69	1.98 +/- 0.83	1.92 +/- 0.70
FT4 (ng/ml) [a]	11.48 +/- 2.05	10.65 +/- 1.58	9.89 +/- 1.09	9.68 +/- 1.42	9.86 +/- 1.46	9.29 +/- 1.74
FT3 (ng/ml) [a]	3.48 +/- 0.54	3.35 +/- 0.41	2.90 +/- 1.00	3.26 +/- 0.75	3.19 +/- 0.67	2.65 +/- 1.11
FT4/FT3 [a]	3.4 +/- 1.01	3.23 +/- 0.76	4.12 +/- 2.34	3.2 +/- 1.15	3.20 +/- 0.66	4.40 +/- 2.06
TSH/FT3 [a]	0.59 +/- 0.20	0.61 +/- 0.23	0.80 +/- 0.57	0.66 +/- 0.33	0.66 +/- 0.39	0.98 +/- 0.67

* male/female; [a]mean, +/- s.d.. All parameters of healthy subjects *vs.* TA patients were compared, for all, $P > 0.05$.