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Original article

A study of the role of DIO1 and DIO2 polymorphism in thyroid cancer and drug response to therapy in the Saudi population

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ABSTRACT

Background: Deiodinases comprise a group of selenoproteins that regulate the bioavailability of active thyroid hormones (TH) in a time and tissue specific fashion. They increase the hormonal activity by metabolizing their inactive precursors to active forms or terminate their activity by deactivating active hormones. The role of the deiodinase (*DIO*) gene polymorphisms in thyroid cancer is not fully understood yet. This study evaluated the potential association of the *DIO1* and *DIO2* genes with differentiated thyroid cancer and differential thyroxine dose requirement in thyroidectomized patients in a Saudi cohort.
Methods: We selected four variants (one *DIO1* and three *DIO2*) for the association studies using Taqman assays in 507 DTC patients undergoing treatment with thyroxin against 560 disease-free individual, all of Saudi Arab origin.
Results: None of the studied variants was linked to differentiated thyroid cancer. The rs1388378_G > T was initially linked to thyroxine dose requirement ($p = 0.035$) when all patients were considered together, but this association was lost when the patients were classified into either near suppressed ($0.1 \leq TSH < 0.5$) or suppressed ($TSH < 0.1$) TSH group.
Discussion: Although the results suggest only a weak relationship with differentiated thyroid cancer, they strongly indicate that the DIO2 polymorphism influences the hormonal dose requirement in patients undergoing treatment with thyroxine. This probably points to a distinction in the way this gene influences disease as compared to therapy thereof.

1. Introduction

Deiodinase (DIOs) are a family of selenoproteins that regulate the bioavailability of active thyroid hormones (THs) in a time and tissue specific manner (Arrojo et al., 2013; Bianco, 2011). These enzymes stimulate the hormone activity by metabolizing inactive precursors to their active forms, and reduce their activity by deactivating them (Bianco and Kim, 2006). Three mammalian deiodinase isozymes D1, D2 and D3 encoded by three deiodinase paralogs *DIO1, DIO2* and *DIO3*, respectively have been described to date (Bianco and Kim, 2006). These membrane-anchored proteins form homodimers to catalyze the removal of iodo groups from the iodothyronine precursors, with different isozymes targeting either the outer or inner-ring groups. Specifically, the D2 is prevalent in cells endowed with the ability to increase T3 levels, therefore enhancing the TH signaling. While it converts the prohormone T4 to the biologically active T3, the D3 in turn metabolizes it to its inactive reverse T3 (rT3) metabolite by removing an iodo group from the inner ring, as well as converting T3 into T2 (Bianco, 2011; Darras et al., 2015). Accordingly, it terminates TH action by inactivating T3 to T2. This combined with the observation that D1 functions as a scavenger by removing iodo groups from both the outer and inner rings means that functional changes in either *DIO1* or *DIO2* genes will trigger perturbations in the hormone metabolism. For example, *DIO1* is thought to influence the

Abbreviations: DIO (1,2,3), deiodinase (1,2,3) gene; D (1, 2), deiodinase (1,2) protein; FT4, free thyroxin; TH, thyroid hormone; TSHβ, thyroid-stimulating homorne-β; T3, triiodothyronine; T4, tetraiodothyronine; UGT1A, UDP glucuronosyltransferase family 1 member A; WSB-1, WB repeat and SOCs box-containing; UDP, uridine phosphorylase.

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well-being in hypothyroid patients (Young Cho et al., 2017), while a number of studies have recently pointed to the probability of *DIO2* polymorphism influencing FT4 metabolism and function (Hoftijzer et al., 2011; Santoro et al., 2014; Torlontano et al., 2008). Thus, a reduction in the negative feedback of FT4 on TSH has been linked to homozygosity for the D2-rs12885300 T allele compared to either the heterozygotes or the wild-types (Hoftijzer et al., 2011), while the need for higher T4 intake in thyroidectomized patients has been linked to its Thr92Ala polymorphism (Torlontano et al., 2008). Put together, these findings point to the likelihood that genic alterations in *DIO2* may predict the T4 requirement to suppress TSH. In this study, we therefore elected to evaluate the role of the *DIO1* and *DIO2* in differentiated thyroid cancer manifestation and possible involvement in the adjustment requirement in treating thyroidectomized patients with the thyroxine (T4).

2. Materials and methods

2.1. Study patients and blood sampling

The study included a total 1067 Saudi individuals comprising 507 patients with differentiated thyroid cancer (DTC) and 560 disease-free controls of Saudi origin. Candidate patients had undergone total thyroidectomy, received radioiodine ablation and were on L-thyroxine suppressive therapy (Euthyrox, Merck Pharmaceuticals, NJ, USA). This therapy aimed to attain either suppressed (TSH < 0.1 mU/L) or near-suppressed (0.1 ≤ TSH < 0.5 mU/L) serum TSH levels with FT4 in the normal range (12–22 pmol/L). The study cases comprised 97.8% papillary thyroid cancer (PTC), 88.9% classic subtype, 9.1% follicular variant PTC, 1.2% tall cell variant, 0.4% diffuse sclerosing subtype, 0.4% insular subtype and 2.2% follicular thyroid cancer. Furthermore, 29.6% of the PTC patients presented with positive family history of the disease. Healthy controls were recruited from the general population and known not to have thyroid disorders, family history of thyroid cancer or to have been previously exposed to therapeutic levels of external radiation. The cases did not differ significantly from the healthy controls in the important confounding variables, such as age and body-mass index (Table 1).

Excluded from the study group were individuals on multiple drug treatment, or those who would have changed the thyroxine brand 3 months prior to the launching of the study. We also excluded patients who might have been on drugs that could potentially interfere with thyroxine treatment. These included (a) anti-epileptics and bile acid resins, (b) thyroid suppressors and other drugs that may alter thyroid hormone metabolism or production, and (c) medicines that could affect the pituitary-thyroid axis. Additionally, expectant females, individuals with mental illness, other types of cancer, as well as those having significant renal impairment (glomerular filtration rate < 60 ml/min) or chronic liver disease were also excluded. Compliance was determined through a questionnaire targeting each subject's medical history, medication use, smoking, as well as measuring the TSH and FT4 levels.

The dose requirement study was directed at the same patient population involved in the disease association study to find the role of gene polymorphism in LT4 dose requirement. Accordingly, 453 out of the 507 DTC patients were included in the analysis. Fifty-four patients were excluded from the analysis because they failed to meet the targeted therapeutic range of TSH and/or FT4 levels [(TSH ≤ 4.3 mU/L); (FT4 = 12.0–22.0 pmol/L)] suggesting non-compliance with thyroxine intake or inappropriate dose prescription. The analysis was performed in two stages. First stage involved whole patient group (ALL) in order to test whether this was general phenomenon with respect to patient response to drug therapy. In the second part, the patients were grouped based on their TSH level into the near suppressed (NSG) (0.1mUI ≤ TSH < 0.5 mU/l) and suppressed (SG) (TSH < 0.1 mU/l) TSH groups to test whether such associations may be related to certain delineable levels of dose required. All participants signed an informed consent approved by the King Faisal Specialist Hospital and Research Centre Ethics Committee, and the study was performed in accordance with the regulations laid down by the Institutional Research Advisory Council in compliance with the Helsinki Declaration principles (http://www.wma.net/en/30publications/10policies/b3/index.html).

2.2. DNA extraction

Genomic DNA was isolated according to the manufacturer's protocol (Gentra Puregene, Qiagen Sciences, Maryland, USA) from 5 ml peripheral blood drawn from each study individual into 6 ml vacutainer tubes containing K_2EDTA (1 Becton Drive, Franklin Lakes, NJ USA). Briefly, 3 ml of blood were added to 9 ml red blood cell lysis buffer and incubated under continuous mixing for 5 min. The mixture was centrifuged at 2000*g* for 2 min, the leucocyte pellet re-suspended and vortexed in 3 ml cell lysis buffer solution and proteins were precipitated for 20 s by centrifuging at 2000*g* for 5 min. The supernatant was mixed with 3 ml isopropanol in a 15 ml tube and genomic DNA precipitated gently. The tube was then centrifuged at 2000*g* for 3 min, the supernatant discarded, and the DNA pellet washed twice in 3 ml of 75% ethanol. The pellet was air-dried, dissolved in 250 µl hydration solution at 65 °C for 1 h, quantified by Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA), aliquoted into 50 µl portions and stored at −20 °C.

2.3. Association studies

The study comprised 507 cases and 560 controls. Primers and TaqMan probes were designed using the Primer Express software V2.0 (Applied Biosystems, Foster City, CA, USA) and procured from Applied Biosystems (Foster City, CA 94404, USA). Genotyping was accomplished by real-time PCR using Taqman chemistry on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc. CA, USA). The fluorogenic probes bearing a suitable reporter dye on the 5′-end and a quencher dye on the 3′-end hybridized to the specific complementary sequence bearing the SNP of interest. Two probes were designed, one labeled with VIC dye and the other with FAM dye at the 5′-primer end. Accordingly, in the process of the primer extension and synthesis of the nascent strand by

Table 1
Demographic data for the individuals in the population-based association study for cancer risk.

	Patients			Controls		
	All (507)	Male (92)	Female (415)	All (560)	Male (159)	Female (401)
Age (years)	45.6 ± 15.0	47.2 ± 15.2	44.6 ± 12.7	45.6 ± 15.2	52.2 ± 15.8	43.0 ± 14.2
BMI	30.3 ± 6.6	28.1 ± 5.7	30.8 ± 6.6	29.5 ± 6.7	28.6 ± 6.0	30.0 ± 7.0
Smoking	36(7.1%)	33(35.9%)	3 (0.7%)	84 (15%)	60 (37.7%)	24 (6.0%)

There was no significant different between the two gender in both age and body mass index. However, there significantly more smokers among the male compared to the females in both the case and controls groups. Age and body mass index (BMI) are given as mean ± standard deviation.

Taq polymerase, the annealed probe is cleaved through exonuclease activity to facilitate the fluorescence emission by releasing the reporter dye from its proximity to the quencher. Optimal working probe concentrations were established by running serial dilutions. A 25 µl master-mix was each prepared by mixing 5 µl of 50 ng DNA, 12.5 µl of 2x Universal mix (Eurogentec, Liege Science Park, 4102 Seraing, Belgium), 1.25 µl of 20x probe assay mix in 6.25 µl DNase-free distilled water. Three controls (without a template) were included in each 96-well plate to normalize the emission signal. For the first cycle, the amplification profile for was set at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 30 sec. The plates were then scanned for FRET signal using the 7900HT sequence detection system and data analyzed using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

2.4. Statistical analysis

Categorical variables were analyzed by Chi-Square test. Genotype and allele comparison between different groups for continuous dependent variables was accomplished by Analysis of Variance (ANOVA) or Student's *t*-test as appropriate, and categorical variables were analyzed by Chi-Square test. Odds ratios and their 95% confidence intervals were computed by logistic regression analysis. Positive associations were entered into multivariate regression analysis with other conventional risk factors for thyroid cancer including age, sex, smoking as predictive variables for the risk of DTC. All other statistical analyses were performed using the SPSS software version 24 (SPSS Inc., Chicago, USA), and data are expressed as mean ± SEM. Associations with a two-tailed *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Association of DIOs with thyroid cancer

In all one *DIO1* SNP rs225013_G > T and three *DIO2* SNPs (rs2294512_A > G, rs1388378_G > T, and rs12885300_C > T) were studied for their association with the disease. The schematic representations of the two genes are given in Figs. 1 and 2. The demographic data of the individuals involved in association analysis are displayed in Table 1. None of the three variants was related in any fashion with thyroid cancer (Table 2).

3.2. Association of DIOs with thyroxin dose requirement

Analysis of the relationship between thyroxine dose requirement and patient response to the therapy was evaluated in 453 DTC patients. The demographic data of individuals involved in the thyroxine dose are displayed in Table 3. The same four variants rs2294512_A > G), rs1388378_G > T, rs12885300_C > T were studied for their possible influence on the variability in thyroxine dose requirement. Initially, the analysis was performed for the whole patient (ALL) group. The results indicated an association for rs1388378_G > T ($p = 0.035$) with dose adjustment requirement. The patients were then grouped into the near suppressed (NSG; $0.1 \leq TSH < 0.5$) and suppressed (SG; $TSH < 0.1$) TSH categories. The rs1388378 lost its association. Furthermore, no significant deviation from the normal doses required for therapy could be established for any of the other variants within a group (Table 4).

4. Discussion

The present study evaluated the potential role of the *DIO1* and *DIO2* gene polymorphisms in thyroid cancer disease as well as in explaining the differences in thyroxin dose requirement by patients undergoing treatment for differentiated thyroid cancer. In all, we elected to investigate one *DIO1* and three *DIO2* variants. None of these SNPs showed any association with the disease. To date, while polymorphisms of deiodinases have been described for some malignancies and several other forms of the diseases, information on their possible involvement in thyroid cancer remains scanty. Specifically, the *DIO2* gene has been associated with diseases such autoimmune hypothyroidism disease (Carle et al., 2017), maternal thyroid status (He et al., 2009), grave's disease (Inoue et al., 2018), drinking behavior (Lee et al., 2015), cognitive impairment (Luo et al., 2015), osteoarthritis (Waarsing et al., 2011; Meulenbelt et al., 2008), but not with thyroid cancer. On the other hand, it is widely acknowledged that, since serum TSH is a sensitive indicator of thyroid function, overt abnormalities in

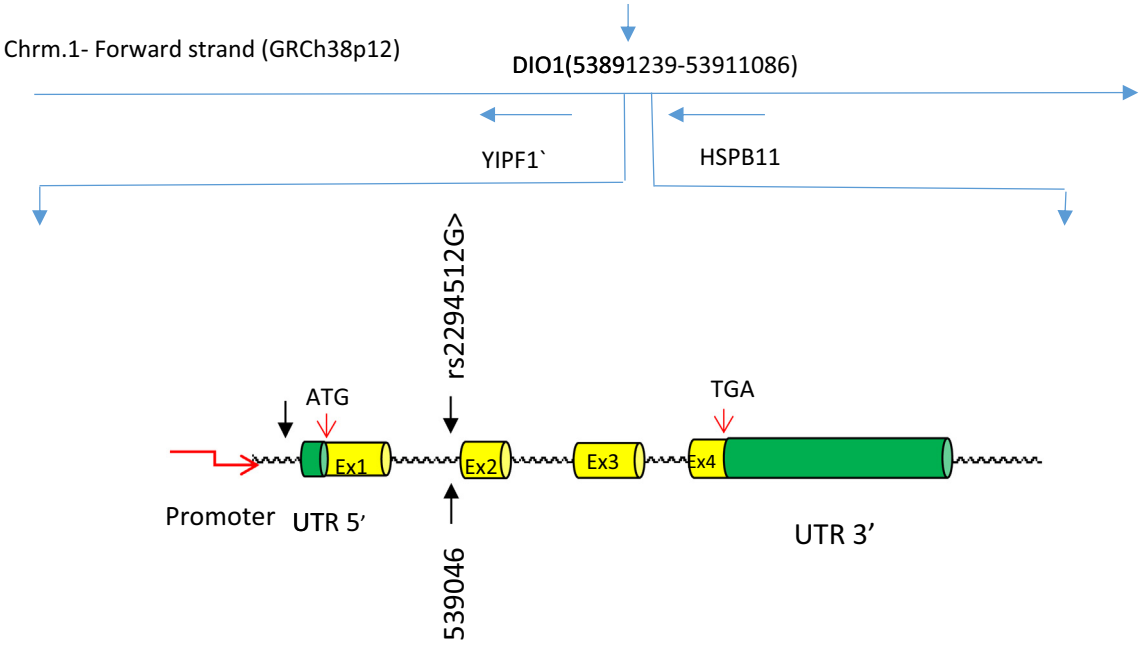

Fig. 1. The figure represents a schematic diagram of the *DIO1* (not to scale) showing the SNPs studied and their chromosomal loci.

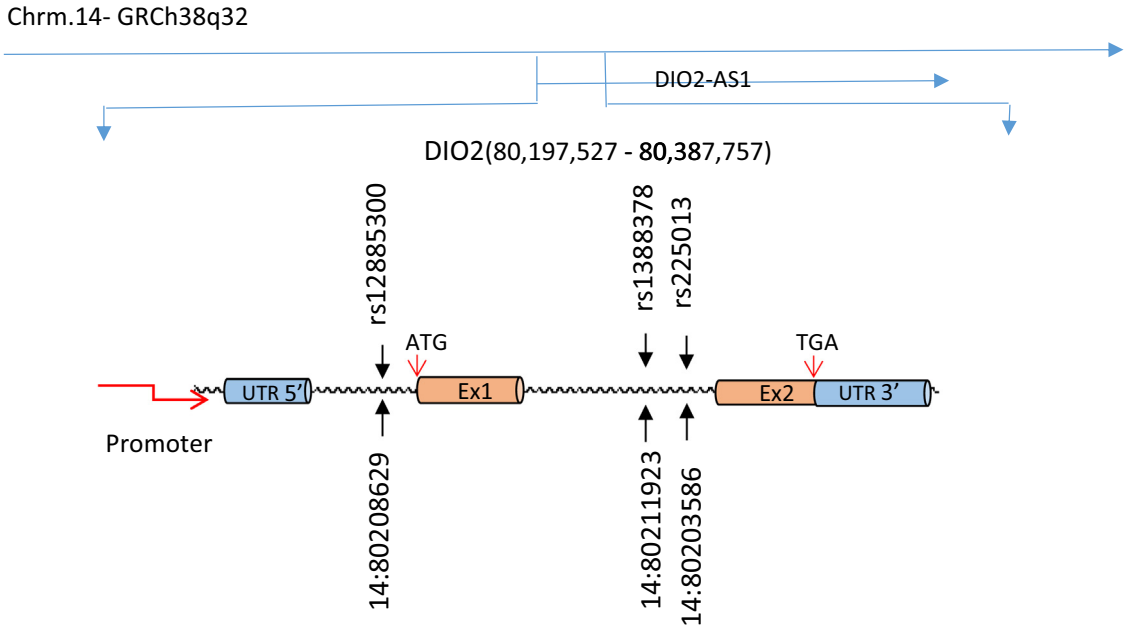

Fig. 2. The figure represents a schematic diagram of the *DIO2*-210 transcript (not to scale) showing chromosomal loci of studied SNPs.

Table 2
Association of *DIO1* and *DIO2* variants with differentiated thyroid cancer risk.

Gene	SNP ID	Genotypes	Patients f(%)	Control f(%)	P	OR(95%CI)
DIO1	rs2294512_A > G	A	252 (25.0)	285 (25.7)	0.701	0.962(0.791–1.171)
		G	758 (75.0)	825 (74.3)		
DIO2	rs1388378_G > T	G	854 (85.4)	925 (85.6)	0.872	0.980(0.768–1.252)
		T	146 (14.6)	155 (14.4)		
DIO2	rs12885300_C > T	C	659 (68.2)	735 (71.6)	0.096	0.850(0.702–1.030)
		T	307 (31.8)	291 (28.4)		
DIO2	rs225013_G > T	G	409 (40.5)	458 (41.0)	0.799	0.978(0.822–1.163)
		T	601 (59.5)	658 (59.0)		

The table compares the real-time PCR data for the 507 studied DTC patients versus 560 healthy controls. SNP ID gives the single nucleotide identification number denoted with the "rs" nomenclature. f(%) gives the frequencies of the genotypes of alleles as a percentage. The letters A, C, G and T are the codes for the nucleotides adenine, cytosine, guanine and thymine respectively. OR, odds ratio; CI, confidence Interval; NS, non-significant; P, P value. Each SNP was entered in a multivariate analysis including age, sex, and smoking.

this function lead to common endocrine disorders affecting a sizeable number of individuals over a life span. These malfunctions are likely to underlie genetic defects. Of the SNPs discussed herein, the rs2294512_A > G of the *DIO1* gene has been found to interact with serum selenium in Crohn patients (Gentschew et al., 2012) and reduced psychological well-being in hypothyroid patients (Young Cho et al., 2017), while the rs1388378_G > T has been linked to mental retardation in the Chinese population (Zhang et al., 2012). Hence, although we were not able to establish any positive relationship for the SNPs with disease per se, genetic alterations in the TSH pathways are likely to play an important role in thyroid cancer manifestation.

Table 3
Demographics data of individuals involved in the thyroxine dose association study.

	All	Male	Female
N	453	82/453(18.1%)	371/453(81.9%)
AGE (years)	45.57 ± 12.90	47.74 ± 14.57	45.09 ± 12.47
BMI	30.34 ± 6.57	28.11 ± 5.72	30.84 ± 6.65
TSH (mU/l)	0.16 ± 0.44	0.21 ± 0.46	0.15 ± 0.43
FT4 (pmol/l)	20.70 ± 1.70	20.69 ± 1.82	20.70 ± 1.67
L-T4dose (μg/kg)	2.05 ± 0.45	2.09 ± 0.51	2.04 ± 0.44

N, number of individuals in the group; BMI, body mass index; TSH, thyroid stimulating hormone; FT4, free thyroxine level; L-T4; Dose is given as μg/kg.

One important subject of interest of the present study was the likelihood that changes in the *DIOs* may explain why DTC patients tend to respond in various fashions to the therapy with thyroid hormones, with some patients requiring thyroxine dose adjustments. When we looked at the cases as a whole, we were able to link these differences to rs1388378_G > T. However, when patients were classified as being in either the near-suppressed or in the suppressed group, this variant lost its link with dose requirement in ether category. Like in the disease manifestation, there is currently lack of information on the role of the *DIOs* in differential response to hormonal therapy of thyroid cancer. On the other hand, the observation that D2 metabolizes T4 into T3 and reverse rT3 into T2, while D1 functions as a scavenger by removing iodo groups means that functional changes in either of the encoding genes is likely to trigger perturbations in the hormone metabolism. To date the genes have been linked to TSH suppression (Santoro et al., 2014), shift in pattern of secretion of thyroid hormone (Peltsverger et al., 2012) and TRH-mediated acute rise in TSH (Luo et al., 2015). Among the variants discussed in the present paper, the rs12885300 is perhaps the most well-studied. It has been linked to TSH suppression (Santoro et al., 2014), TRH-

Table 4
Influence of DIO1 variant (rs2294512_A > G), DIO2 variants (rs1388378_G > T, rs12885300_C > T) and TSHB variants (rs201857310_A > G, rs7530810_A > G) on thyroxine dose requirement.

			All (TSH < 4.3)			NSG 0.1 ≤ TSH < 0.5			SG(TSH < 0.1)		
Gene	SNP ID	Geno-types	N	T4 dose	P	N	T4 dose	P	N	T4 dose	P
DIO1	rs2294512_A > G	A	245	150.7 ± 32.3	0.78	59	149.4 ± 34.3	0.92	172	151.0 ± 31.0	0.78
		G	735	151.4 ± 33.4		213	149.9 ± 33.1		466	151.8 ± 32.5	
DIO2	rs1388378_G > T	G	828	152.2 ± 33.1	*0.035	229	150.9 ± 33.1	0.24	535	152.4 ± 32.2	0.17
		T	144	145.9 ± 33.3		35	143.7 ± 36.7		103	147.6 ± 31.5	
DIO2	rs12885300_C > T	C	644	152.2 ± 32.8	0.29	183	151.3 ± 34.9	0.097	417	152.5 ± 31.6	0.76
		T	296	149.8 ± 34.0		75	143.7 ± 29.1		195	151.6 ± 33.6	
DIO2	rs225013_G > T	G	399	151.6 ± 33. 6	0.82	116	149. 8 ± 35.7	0.92	255	152.1 ± 31.7	0.78
		T	581	151.2 ± 32. 8		154	150.2 ± 31.6		385	151.4 ± 32.3	

mediated acute rise in TSH (Luo et al., 2015), a shift in pattern of secretion of thyroid hormone (Peltsverger et al., 2012) and TRH-mediated acute rise in TSH (Luo et al., 2015). Specifically, structural alterations in the *DIO2* have been implicated in differential response to disease therapy (He et al., 2009). One study suggested that negative feedback of FT(4) on TSH was weaker in patients homozygous for the D2-rs12885300 T allele than in wild-type and heterozygous subjects (Hoftijzer et al., 2011). Furthermore, the Thr92Ala polymorphism was suggested to predict the need for higher T4 intake in thyroidectomized patients (Torlontano et al., 2008). Besides, UGT1A polymorphism has been associated with T4 dosage in DTC patients, but the effect appeared to account for only a very small proportion of individuals (Santoro et al., 2014). Ubiquitination is an essential step in the control of D2 activity by triggering its inactivation through T4 binding to and/or T4 catalysis mediated by the E3 ubiquitin ligases WSB-1 and or TEB (Arrojo et al., 2013). Therefore, UGT polymorphism is also likely to influence the D2 function, thereby triggering alterations in the thyroid stimulating hormone levels. Thus, while no speculation can be advanced based on these findings alone, it can nonetheless be suggested that this may be related to gene regulatory mechanisms of the regulation of the bioavailability of the hormone.

5. Conclusion

Although it remains unclear as to whether *DIO* polymorphism exerts direct impact on thyroid cancer disease manifestation, it can be speculated that *DIO2* plays an important role in determining thyroxine dose requirement in patients under hormonal therapy for differentiated thyroid cancer, which calls for further investigation to discern this link more clearly.

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