Association of Type II 5' Monodeiodinase Thr92Ala Single Nucleotide Gene Polymorphism and Circulating Thyroid Hormones Among Type 2 Diabetes Mellitus Patients

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Abstract Diabetes mellitus and thyroid disorders are common endocrinopathies, which often occur parallel. Dyslipidemia is very common in both of these conditions. The development of hypothyroidism is well-known in type 1 diabetics, but it was not distinctly understood in type 2 diabetics. Thus we tried to examine the association between type II deiodinase (D2 or DIO2) Thr92Ala single nucleotide gene polymorphism and thyroid function among type 2 diabetes mellitus patients. A total of 130 type 2 diabetics were screened and genotyped for DIO2 Thr92Ala polymorphism. Fasting plasma glucose, Glycosylated haemoglobin, lipid and thyroid profiles, malondialdehyde (MDA) and paraoxonase were estimated according to standard procedures. A significant altered level of thyroid hormones (TH's) was found in Ala/Ala genotype when compared with Thr/Thr or Thr/Ala genotype. DIO2 and T₃:T₄ ratio significantly decreased, whereas total T₄ and thyroid stimulating hormone levels significantly elevated among Ala/Ala genotype (131 ± 30 ng/ml; 0.12 ± 0.05 ; 7.17 ± 2.05 µg/dl; 4.77 ± 3.1 µIU/ml, respectively) when compared with Thr/Thr + Thr/Ala genotypes (176 ± 33 ng/ml; 0.21 ± 0.05 ; 5.21 ± 1.1 µg/dl; 2.59 ± 1.61 µIU/ml respectively). Moreover, D2 levels were significantly negatively correlated with TH's levels except total T₄ among Ala/Ala genotypes. All the patients were having a poor glycemic control, and their glycemic status was positively correlating with MDA levels. On the other hand,

serum paraoxonase activity decreased among Ala/Ala genotype (104 ± 21 vs. 118 ± 18 nmol/min/ml). In conclusion, DIO2 Ala92 homozygous variant found to be associated with altered levels of DIO2, Thyroid profile and paraoxonase. Hence, we recommend to do detail study of genetic factors related to thyroid function and prevent additional diabetic complications.

Keywords DIO2 Thr92Ala · Type II 5' monodeiodinase · Thyroid hormones · Paraoxonase · Type 2 diabetes mellitus

Introduction

The physiological proportions of thyroid hormones (TH's) are very essential for maintaining internal milieu in both health and disease of human beings. The levels of circulating TH's were regulated by hypothalamic pituitary thyroid axis (HPTA) and vice versa. The imbalance in the TH's is due to either inefficiency of HPTA or insufficient production of TH's. Thyroid disease, especially hypothyroidism is often encountered in type 2 diabetics, which deteriorate and progress the disease by exaggerating the diabetic complications such as dyslipidemia, obesity, insulin resistance and atherosclerosis [1].

3, 5, 3'-L-triiodothyronine (T₃) is a biologically active hormone, which is required for maintaining energy, lipid and protein metabolism. T₃ exerts its actions at the cellular level through the activation of mRNA coding for specific proteins; such as HMG-CoA reductase, cholesterol ester transfer protein (CETP), lecithin cholesterol acyl transferase, hepatic lipase (HL), lipoprotein lipase (LPL) etc., via binding to specific nuclear receptors [2]. There is an elevated level of triacyl glycerol (TAG), total cholesterol (TC) and low-density lipoprotein cholesterol (LDLc) and

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decreased/normal levels of high-density lipoprotein cholesterol (HDLc) have been observed in the hypothyroid state. The T_3 is produced from prohormone 3, 3', 5, 5'-L-tetraiodothyronine (Thyroxine/ T_4) by the process called as outer ring deiodination. This process is carried by three types of selenium dependent metalloproteins, and collectively called as 'monodeiodinases'. Two types of deiodinations are possible, namely outer ring (5') and inner ring deiodination (5) of T_4 . Outer ring deiodination of T_4 is carried by both Type I monodeiodinase (D1) and Type II monodeiodinase (D2), which supplies 80 % of circulating T_3 in humans [3]. D1 is the only selenodeiodinase which catalyzes both outer and inner ring deiodination. It is found in liver, kidney, thyroid, and pituitary etc. D2 is exclusively deiodinates only at outer ring; it is abundantly found in tissues such as thyroid, heart, brain, spinal cord, skeletal muscle, and placenta. It contributes to the maximum amount of circulating T_3 . Since, it has a low K_m for T_4 (~ 2 nm), about three orders of magnitude lower than that of D1 under similar in vitro conditions. Type III monodeiodinase (D3) exclusively deiodinate the inner ring of T_4 and is converted into reverse T_3 (rT_3), which is biologically inactive [4].

The balanced activities of all these three deiodinases are essential to maintain the proportion of T_3 to T_4 , and later in turn are essential for controlling HPTA loop. Any changes in these catalytic proteins lead to changes in TH levels. Single nucleotide gene polymorphisms (SNP's) were considered as non fatal mutations which occurs on an average once per 250–1000 bp and account for ~ 90 % of DNA sequence variants in the human genome [5]. The high density and mutational stability of SNP's make them particularly useful DNA markers for population genetics and for mapping susceptibility genes for complex diseases [6]. To date, there are several SNP's were found and some of them were may interfere with the phenotypic manifestation of these enzymes influencing the levels of thyroid hormone.

Threonine at 92 position of type II 5' monodeiodinase is replaced by Alanine due to a missense mutation of *Adenine* by *Guanine* at 274th codon (rs225014) in exon2 of DIO2 gene [7]. The commonly occurring Thr92Ala SNP was found to be associated with obesity and insulin resistance. The D2 activity found to decline among Ala92Ala mutant homozygous when compared to Thr/Thr wild homozygous and Thr/Ala heterozygous genotypes [8]. The DIO2 Thr92Ala polymorphism has also been linked to increased risk for osteoarthritis [9], hypertension [10], Graves' disease [11], intelligence quotient alterations associated with iodine deficiency [12], psychological well-being and response to T_3 or T_4 treatment [13], and decreased bone mass and higher bone turnover [14]. Intriguingly, most of these associations are independent of serum thyroid

hormone levels, which highlight the importance of local regulation of thyroid hormones in peripheral tissues.

In view of the relatively high prevalence of both endocrinopathies, it is important to investigate all diabetic patients for thyroid disorders. In spite of the perpetual monitoring and efforts towards new therapeutics, diabetic associated complications remain a major health problem. Therefore, we sought to study the association between commonly occurring D2 Thr92Ala polymorphism and thyroid function among type 2 diabetes mellitus patients.

Methods and Materials

In the present study, we included 130 non-smoker and non-alcoholic male type 2 diabetes mellitus subjects who were attending diabetic outpatient department of Rajiv Gandhi Govt. General Hospital, Chennai, India. All subjects were genotyped for DIO2 Thr92Ala genotype (rs225014) by using tetra primer amplification refractory mutation system polymerase chain reaction (TP-ARMS-PCR). Informed consent was obtained from all subjects before enrollment. This study was approved by the institutional ethics committee.

Sample Collection

7 ml of fasting venous blood sample was collected separately in both K_2 EDTA and clot activator Becton–Dickinson (BD) vacutainer tubes. Required quantity of whole blood, serum and plasma were aliquoted in order to avoid repeated freezing and thawing effect on measuring analytes and kept at -40 °C until analysis.

Biochemical Procedures

Fasting plasma glucose (FPG), lipid profile parameters were estimated by using commercial assay kits (Spinreact, SA, Santa Coloma, Spain). Glycosylated haemoglobin (HbA_{1c}) was estimated in whole blood by the ion exchange resin method provided by Diatek, Kolkata. A thyroid profile includes thyroid stimulating hormone (TSH), Total T_3 , Free T_3 , Total T_4 and Free T_4 was measured by using commercial enzyme linked immuno sorbent assay (ELISA) kits (BeneSphera™/Avantor performance materials Ltd. USA). Serum levels of Type II 5' monodeiodinase (D2) was measured by commercially available ELISA kits (Qayee-Bio, Shanghai, China).

Basal Paraoxonase (bPON) Activity

The basal arylesterase activity of paraoxonase (E.C.3.1.1.2; basal PON) was measured spectrophotometrically as

described previously [15]. In brief, to the 50 μL of serum 3 ml of tris buffer (20 mM) was added and initial absorbance was adjusted to 0.5 in spectrometer at a wavelength of 412 nm. The reaction was initiated by adding 50 μL of 5.5 mM p-nitrophenyl acetate as substrate. The rate of increase in absorbance (A) was monitored for 2.5 min. The same protocol was followed in order to determine the rate of non-enzymatic hydrolysis. The corrected 'A' was obtained by subtracting the nonenzymatic ΔA from the total ΔA . PON activity was calculated by using a molar extinction coefficient of $17,000 \text{ M}^{-1} \text{ cm}^{-1}$. PON activity expressed as nmol PON/min/ml of serum.

Malondialdehyde (MDA)

Thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxide (malondialdehyde) were measured spectrophotometrically by using the method described by Draper and Hadley [16]. Briefly, 500 μL of protein free filtrate prepared in 24 % trichloro acetic acid (TCA) was taken and 125 μL of freshly prepared thiobarbituric acid (TBA) was added. The tubes were mixed, sealed and incubated at 100°C for 60 min. Then the tubes were cooled and 500 μL of butanol was added. Then the tubes were centrifuged for 10 min at 2000 rpm. The OD of supernatant was measured at 532 nm by a spectrophotometer [16].

Calculation = OD of sample/ 1.56×105 . The concentration of plasma MDA was expressed as $\mu\text{mol/L}$.

Molecular Methods

Materials

DNA purification kit (PureFast[®]), PCR Master Mix for DNA isolation and TP-ARMS-PCR, Agarose gel electrophoresis consumables and primers purchased from HELINI Biomolecules, Chennai, India.

Primers

Primers were designed by using http://cedar.genetics.soton.ac.uk/public_html/primer1 database software; this software was designed by Ye et al., Human Genetics Research Division, University of Southampton, UK.

Primer (A allele)

FW: ATTGCCACTGTTGTACCTCCTTCGGT
Rv: CTATGTTGGCGTTATTGTCCATGCGGTC

Primer (G allele)

FW: AATTCCAGTGTGGTGCATGTCTCCATTG
Rv: TTTTGGGCCATTCTTTACATTACCTGCCA

DIO2 Thr92Ala (c. 274A > G; p. T92A; rs225014) SNP Genotype Analysis

Method

Tetra Primer Amplification Refractory Mutation System Polymerase Chain Reaction (TP-ARMS PCR).

Principle

Two allele-specific amplicons are generated using two pairs of allele specific primers. In a single step reaction, the outer primers amplify a large fragment of the target gene, irrespective of its genotype although each inner primer combines with a particular opposite outer primer to generate smaller allele-specific amplicons, which are of different sizes and can easily be discriminated on gel electrophoresis either as homozygous or heterozygous. Allele specificity is improved by introducing a mismatch nucleotide at position 2 from 3' terminal of an inner primer and in the template DNA strand. The primers are 26 NT or longer, so as to minimize the difference in stability of primers annealed to the target and non-target alleles, ensuring that allele specificity results from differences in extension rate, rather than hybridization rate. By placing the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length, allowing them to be discriminatory by the gel electrophoresis [17].

PCR Procedure

Each PCR reaction was carried out in a total volume of 30 μL , containing 2 μL of template DNA, 2 μL of primer mix (5 pmol/ μL), 20 μL master mix and 6 μL of water (nuclease free). The solution was overlaid with 5 μL of liquid paraffin and incubated for 3 min at 95°C , followed by 30 cycles of 30 Sec denaturation (95°C), 30 Sec annealing (60°C) and 30 Sec extension (72°C) and an additional 5 min extension at 72°C at the end of the 30 cycles.

A 30 μL aliquot of the PCR products was mixed with 8 μL 6 $\times$ Gel loading dye and subjected to apply in 2 % agarose gel (in 1 $\times$ TAE buffer). Run electrophoresis at 50 V till the dye reaches three fourth distances and observe the bands in UV Transilluminator (Fig. 1).

Fig. 1 Agarose gel electrophoresis of DIO2 Thr92Ala SNP genotypes by TP-ARMS-PCR

- Product size for A allele: 276 bp
- Product size for G allele: 418 bp
- Product size of two outer primers: 639 bp

Statistical Analysis

Mean $\pm$ 2SD were used to describe various parameters in the study groups. One-way analysis of variance (ANOVA) was used to compare the mean difference between study groups. Karl Pearson correlation was also used to determine the trend between various parameters. A simple linear regression analysis was performed between selected dependant and predictor variables within the groups. A two tailed t test $p < 0.05$ was considered as statistically significant. χ^2 test $p > 0.05$ was considered for allelic frequency in consistent with Hardy–Weinberg Equilibrium (HWE). All the statistical parameters were analyzed by using MedCalc 15.4 Bvba and STATISTICA 7.0 (StatSoft Inc., USA) software packages.

Results

Genotype and allelic frequencies of the DIO2 Thr92Ala SNP among type 2 diabetics were shown in Table 1. The allelic frequencies were consistent with HWE (χ^2 test, $p = 0.46$) among the study groups. The mean $\pm$ SD of various study parameters among the genotypes were described in Table 2. Among the various biochemical parameters studied, serum levels of D2, Thyroid Hormones

and basal activity of paraoxonase were significantly altered among Ala/Ala genotype when compared with the Thr/Thr or Thr/Ala genotypes (Table 2). However, mean $\pm$ SD of Age, BMI, FBG, HbA_{1C}, MDA and lipid profile parameters were not statistically differed between genotypes (Table 2). Hence we considered *recessive inheritance model* (Thr/Thr + Thr/Ala Vs Ala/Ala) to find the effect of Ala homozygosity on D2 activity and thyroid function (Table 2). One-way ANOVA was performed to determine the mean difference of various parameters between Thr/Thr + Thr/Ala and Ala/Ala genotypes (Table 2).

All the patients were having a poor glycemic control and their glycemic status was positively correlating with MDA levels. Lipid profile parameters showing normal pattern among the groups, however total HDLc was falling to lower limit of reference range (Table 2). Serum paraoxonase activity was significantly decreased among Ala/Ala homozygous variants (104 ± 21 nmol/min/ml) when compared with Thr/Thr or Thr/Ala genotypes (118 ± 17 & 118 ± 18 nmol/min/ml respectively, $p < 0.0001$).

The levels of D2 is low among Ala/Ala homozygote's when compared with Thr/Thr or Thr/Ala genotypes (131 ± 30 vs. 169 ± 36 vs. 179 ± 32 ng/ml, $p < 0.0001$, Table 2). Thyroid profile parameters were in reference range among the three genotype groups, but the Ala homozygous found to be altered levels when compared with Thr/Thr or Thr/Ala genotypes. Total T₄, free T₄ and TSH levels were raised to an upper normal range (7.17 ± 2.05 μ g/dl; 1.14 ± 0.24 ng/dl and 4.77 ± 3.1 μ IU/ml, respectively), whereas Total T₃, free T₃ levels were close to the lower reference range (0.81 ± 0.15 ng/ml & 1.82 ± 0.35 pg/ml, respectively) among Ala/Ala genotypes (Figs. 2, 3, 4, 5, 6). The ratio of T₃ to T₄ much decreased in Ala/Ala genotypes when compared with Thr/Thr or Thr/Ala genotypes (0.12 ± 0.05 vs. 0.21 ± 0.046 vs. 0.215 ± 0.053 respectively, $p < 0.0001$).

Simple linear regression analysis was performed between selected dependant and predictor variables (Table 3; Figs. 7, 8, 9, 10, 11, 12, 13, 14, 15, 16). The coefficient of determination (R^2) for all the TH's found to statistically significant (Table 3) among the genotypes. TSH levels were significantly negatively regressing with D2 levels in all the genotypes. Serum T₃:T₄ ratio significantly positively regressing with D2 levels among Thr/

Table 1 Showing genotype and allelic frequency of Type II DIO2 gene SNP at Thr92Ala

Subjects	Genotype frequencies (n and %)			Allele frequencies (%)	χ^2 -test p value
	Thr/Thr	Thr/Ala	Ala/Ala		
Cases (n = 130)	22 (16.9 %)	58 (44.6 %)	50 (38.5 %)	Thr-39 Ala-61	0.46*

* Indicates the allelic frequencies were consistent with HWE

Table 2 Showing comparison of various study parameters among genotypes

Parameter	Thr/Thr (n = 22)	Thr/Ala (n = 58)	Thr/Thr + Thr/Ala (n = 80)	Ala/Ala (n = 50)	ANOVA p value
Age (Y)	58 ± 11	60 ± 9	59 ± 10	63 ± 9	0.12
BMI	25 ± 4	25 ± 5	25 ± 4	24 ± 2	0.54
FBG (mg/dl)	137 ± 40	151 ± 61	148 ± 56	138 ± 58	0.33
HbA _{1C} (%)	7.52 ± 0.98	7.51 ± 1.04	7.5 ± 1	7.65 ± 1.09	0.78
TAG (mg/dl)	145 ± 97	117 ± 72	125 ± 81	131 ± 34	0.20
TC (mg/dl)	152 ± 19	158 ± 94	157 ± 80	161 ± 40	0.87
LDLc (mg/dl)	83 ± 30	87 ± 33	86 ± 32	93 ± 26	0.38
HDLc (mg/dl)	46.6 ± 4.12	46.9 ± 5.05	46 ± 5	46.7 ± 5.2	0.60
bPON(nmol/min/ml)*	118 ± 17	118 ± 18	118 ± 18	104 ± 21	0.000
MDA (μmol/l)	13 ± 5	11 ± 6	12 ± 6	11 ± 4	0.505
TSH (μIU/ml)*	2.88 ± 1.92	2.48 ± 1.48	2.59 ± 1.61	4.77 ± 3.1	0.000
Total T ₃ (ng/ml)*	1.09 ± 0.12	1.07 ± 0.13	1.07 ± 0.13	0.81 ± 0.15	0.000
Free T ₃ (pg/ml)*	2.02 ± 0.53	2.2 ± 0.31	2.16 ± 0.39	1.82 ± 0.35	0.000
TT ₃ :TT ₄ ratio*	0.21 ± 0.046	0.215 ± 0.053	0.21 ± 0.05	0.12 ± 0.05	0.000
Total T ₄ (μg/dl)*	5.26 ± .91	5.2 ± 1.17	5.21 ± 1.1	7.17 ± 2.05	0.000
Free T ₄ (ng/dl)*	0.98 ± 0.26	1.11 ± 0.25	1.07 ± 0.26	1.14 ± 0.24	0.031
D2 (ng/ml)*	169 ± 36	179 ± 32	176 ± 33	131 ± 30	0.000

* Indicates the significance difference between Ala/Ala and Thr/Thr or Thr/Ala genotypes

Fig. 2 Error bar plot for D2 among genotypes

Thr + Thr/Ala genotypes, whereas it was found to be significantly negative among Ala/Ala genotype. The coefficient of determination of T₃:T₄ ratio was significant among Thr/Thr + Thr/Ala genotypes ($r = -0.42$, $R^2 = 0.175$), but it was not significant among Ala/Ala genotypes ($r = 0.05$, $R^2 = 0.003$), when we consider TSH as an independent variable.

Fig. 3 Error bar plot for TSH among genotypes

Discussion

Diabetes mellitus is a contributing cause of morbidity & mortality all over the world. Altered quantity and quality of the lipid and lipoproteins (dyslipidemia) was well studied in diabetics and it was set up to be an important risk factor for growth of CVD risk and death. Thyroid disorders such as subclinical hypothyroidism may occur in diabetics, which will exacerbate the coexisting dyslipidemia and

Fig. 4 Error bar plot for $T_3:T_4$ among genotypes

Fig. 5 Error bar plot for HDLc among genotypes

Fig. 6 Error bar plot for basal paraoxonase among genotypes

further increase the risk of cardiovascular diseases [18]. Development of hypothyroidism in type 1 diabetics was secondary to the autoimmune destruction of beta cells; studies found that, it is partly due to the polyglandular autoimmune syndrome, [19] whereas it was not clearly understood among type 2 diabetics. It is postulated that subclinical hypothyroidism is more prevalent in metabolic disease patients [20].

The physiological levels of TH's were efficiently regulated by HPT axis. HPT axis which includes hypothalamus, thyrotrophic releasing hormone (TRH) and TSH were finely controlled by TH's via negative feedback mechanism. Thus, the physiologically available bio-active T_3 is very important to accelerate the HPTA loop. The majority of circulating T_3 is produced in extrathyroidal tissues by the process called as extrathyroidal 5' monodeiodination (Outer ring) of prohormone T_4 . The outer ring monodeiodination is carried by two selenodeiodinases, namely D1 & D2. The major role of the D2 is to control the intracellular T_3 concentration, its accessibility to the nucleus, and the saturation of the nuclear T_3 receptor in target tissues [4]. Moreover, D2 is likely to protect tissues from the detrimental effects of hypothyroidism because its low K_m (10^{-9}) continues to permit the efficient local conversion of T_4 to T_3 [21]. Hence, any alteration in these enzyme activities may affect the TH's levels.

Subclinical hypothyroidism (SCH) is characterized as normal/sub normal levels of TH's and but slightly elevated level of TSH. The blood concentration of TSH is increased due to suppressed negative feedback from TH's. Analysis of the HPT axis in D2 knockout mice demonstrated a 2-3 fold increase in the circulating TSH concentration and 27-40 % increase in the T_4 level accompanied by reducing clearance of T_4 from plasma, but at normal levels of T_3 [22–24]. Hypothyroidism in diabetics induces the secondary dyslipidemia; it is characterized as elevated levels of TAG, TC, LDL and altered levels of HDL. Among HDL subfractions, HDL₂ is elevated, since HL, LPL, CETP etc., were up regulated by T_3 . Thus, the quantity and quality of lipid and lipoproteins are drastically affected during hypothyroid state [25].

In the present study, we observed statistically significant difference in D2 and TH levels between the Thr/Thr + Thr/Ala and Ala/Ala genotypes, although there is a normal pattern of thyroid profile (Table 2). TSH levels were normal in all the groups, but a significant rise seen in Ala/Ala genotypes (4.77 ± 3.1 μ IU/ml) when compared with Thr/Thr and Thr/Ala genotypes (2.88 ± 1.92 & 2.48 ± 1.48 μ IU/ml respectively). Moreover, TSH levels significantly negatively correlated with D2 levels in all the

Table 3 Simple linear regression analysis between selected dependant and predictor variables by assuming recessive inheritance model

Constant	D.V	R		R ²		Adj. R ²		SEE	
		A/A + A/G (n = 80)	G/G (n = 50)	A/A + A/G	G/G	A/A + A/G	G/G	A/A + A/G	G/G
D2	TSH	−0.62*	−0.61*	0.379	0.372	0.371*	0.359*	1.27762	2.48566
	T ₃ :T ₄	0.51*	−0.38*	0.261	0.145	0.251*	0.127*	0.0444	0.04679
TSH	T ₃ :T ₄	−0.42*	0.052	0.175	0.003	0.164*	−0.018	0.04692	0.05052

D.V dependent variable, R correlation coefficient, R² coefficient of determination; SEE standard error of the estimate

* Indicates the p value is < 0.05

Fig. 7 Scatter plot of TSH against D2 among Thr/Thr + Thr/Ala genotypes **Fig. 9** Scatter plot of Total T₄ against D2 among Thr/Thr + Thr/Ala genotypes **Fig. 8** Scatter plot of Total T₃ against D2 among Thr/Thr + Thr/Ala genotypes **Fig. 10** Scatter plot of ratio of T₃:T₄ against D2 among Thr/Thr + Thr/Ala genotypes

genotypes. The ratio of T₃:T₄ was showing significant positive correlation with D2 levels among Thr/Thr and Thr/Ala genotypes ($r = 0.51$; $p < 0.05$), whereas this was a significant negative in Ala/Ala genotype ($r = -0.38$, $p < 0.05$).

However, serum levels of T₃ and fT₃ were subnormal in Ala/Ala genotype (1.82 ± 0.35 ng/ml and 0.12 ± 0.05 pg/ml respectively, $p < 0.05$). Therefore, from the study results it is clear that, Ala/Ala genotype is associated with decreased

Fig. 11 Scatter plot of TSH against D2 among Ala/Ala genotypes

Fig. 14 Scatter plot of T₃:T₄ ratio against D2 among Ala/Ala genotypes Fig. 12 Scatter plot of Total T₃ against D2 among Ala/Ala genotypes Fig. 15 Scatter plot of T₃:T₄ ratio against TSH among Thr/Thr + Thr/Ala genotypes Fig. 13 Scatter plot of Total T₄ against D2 among Ala/Ala genotypes Fig. 16 Scatter plot of T₃:T₄ ratio against TSH among Ala/Ala genotypes

quantity and quality of D2, which in turn have a substantial effect on thyroid hormone concentrations. Our results were agreeing with previous studies. Canani et al. demonstrated a decreased enzyme velocity in Ala homozygous variants in their *ex vivo* studies [8]. In the study of JM Dora showed that, the Ala mutant variant induces the insulin resistance, since most of the subjects are found to be Ala/Ala genotype [7]. We also performed linear regression analysis between selected dependent and predictor variables (Table 3; Figs. 7, 8, 9, 10, 11, 12, 13, 14, 15, 16). The coefficient of determination for TSH & T₃:T₄ ratio was found to be statistically significant with D2 levels among the Thr/Thr + Thr/Ala and Ala/Ala genotypes (Table 3; Figs. 7, 8, 9, 10, 11, 12, 13, 14, 15, 16; $p < 0.05$). In normal circumstances, D2 is immediately ubiquitinated and degraded in the presence of T₄. Therefore D2 has a less half life about 20 min in the presence of T₄, which is relatively lesser than D1 (12 h) [4]. In our study, total T₄ levels are significantly increased in Ala homozygous variant, moreover, which is negatively correlated with D2 levels. Therefore, the decrease in T₃:T₄ ratio would decrease the circulating D2. These observations confirm that, there is an altered phenotype expression of the DIO2 gene in the Ala/Ala genotype and lead to the development of hypothyroidism. Butler et al. showed a delayed T₃ production in Ala92 homozygous subjects after TRH stimulated TSH secretion [26]. Recently, Torlontano et al. demonstrated that athyreotic patients with Ala/Ala genotype require higher doses of T₄ replacement therapy to achieve near suppression of serum TSH [27]. These findings suggest that there is a reduced D2 activity among Ala/Ala homozygous pituitary thyrotrophs. On the other hand, Heemstra et al. showed that, DIO2 92Ala homozygous variants not responded to higher doses of T₄ replacement therapy in either athyroid patients or Hashimoto thyroiditis based on their regression analysis model [28]. However, our study is the first study in India to describe an association between Thr92Ala SNP in DIO2 gene and Thyroid function among type 2 diabetes mellitus. The present study has certain limitations and it is the first preliminary report on South Indian type 2 diabetes mellitus population to examine the association between Thr92Ala SNP in DIO2, and Thyroid function among diabetics, therefore large sample prospective study may require for strengthening the results.

We also measured lipid peroxidation end product such as malondialdehyde (MDA). MDA irreversibly binds various biomolecules and damages them. Hyperglycemia is known to be induced oxidative stress, which in turn leads to lipid peroxidation and formation of lipid peroxides such as MDA [29]. In the present study, all the subjects were under poor glycemic control (Table 2; FBS = 144 ± 56 mg/dl; HbA_{1c} = 7.57 ± 1.04 %). Moreover, there was no significant change in the plasma MDA levels in the study population, but which is holding significant positive correlation with glycemic status.

We also measured serum paraoxonase activity to determine anti atherogenic properties of HDL. Paraoxonase (PON) is a Ca²⁺ dependent aryl esterase synthesized and secreted by the liver [30, 31]. It is associated with ApoA1 of HDL complex and hydrolyzes various toxic compounds which include organophosphates, aryl esters, oxidized phospholipids and lipid peroxides. Several studies found that the activity of PON is decreasing during oxidative stress, acute myocardial infarction [32], familial hypercholesterolemia, and in diabetes mellitus [33]. Thus, the low serum PON activity has been implicated in the development of coronary heart diseases in diabetes. However, there is no such a study to describe the association of PON activity and thyroid dysfunction. In the present study, serum PON activity is significantly decreased in Ala/Ala genotypes when compared with Thr/Thr or Thr/Ala genotypes, but the levels are not correlating with HDL concentration (Table 2). The similar results were shown in our previous study [34]. The decrease in serum PON activity among Ala homozygous was not dependent on HDL concentration, would explain the importance of quality of HDL rather than its concentration. Therefore, thyroid dysfunction may have an effect on the anti atherogenic function of HDL.

Conclusion

From the study results it is concluded that, Ala/Ala homozygous variant is showing reduced levels of D2 and altered levels of TH's when comparing with Thr/Thr or Thr/Ala genotypes. The increasing in TSH levels and decrease in T₃:T₄ ratio, thus confirms, there is an intrinsic thyroid disease among Ala mutants. Ala homozygosity also associated with reduced activity of paraoxonase. Hence, we recommend to do detailed study of genetic factors related to thyroid function and prevent additional diabetic complications.

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Compliance with Ethical Standards

Conflict of interest All authors involved in this work have declared that 'There is no conflict of interest'.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving human participants and/or animals All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later

amendments or comparable ethical standards. Ethics committee certificate No. 18062011, Madras Medical College, Chennai-3.

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