A new polymorphism in the type II deiodinase gene is associated with circulating thyroid hormone parameters

Robin P. Peeters,1 Annemieke W. van den Beld,1 Hayat Attalki,1 Hans van Toor,1 Yolanda B. de Rijke,1,2 George G. J. M. Kuiper,1 Steven W. J. Lamberts,1 Joop A. M. J. L. Janssen,1 Andre G. Uitterlinden,1,2,3 and Theo J. Visser1
Departments of 1Internal Medicine, 2Clinical Chemistry, and 3Epidemiology and Biostatistics, Erasmus University Medical Center, Rotterdam, The Netherlands
Submitted 3 December 2004; accepted in final form 15 February 2005

THYROID HORMONE IS ESSENTIAL for growth, development, and regulation of energy metabolism (14). It stimulates metabolic rate by increasing ATP turnover and by regulating the expression of uncoupling proteins in the mitochondria of fat and skeletal muscle (26, 33). Production of thyroid hormone is regulated by the classic hypothalamus-pituitary-thyroid axis, whereas the biological activity of thyroid hormone, i.e., the availability of triiodothyronine (T3), is mainly regulated by the three different selenodeiodinases (D1-D3) (2, 15). Tissue distribution and enzymatic activity of the three deiodinases are highly specific, and they all play a different physiological role. D2 is present in brain, pituitary, thyroid, brown adipose tissue, skeletal muscle, and aortic smooth muscle cells in humans (2, 6, 15, 17, 20, 30, 31). It converts thyroxine (T4) to T3 by outer-ring deiodination, and it is important in the regulation of local thyroid hormone bioactivity in D2-expressing tissues. D2 in skeletal muscle may also contribute to serum T3 production (2, 30).

The mRNA of the D2 gene is unusually long (6–7 kb) compared with the other two selenodeiodinases, containing long 5′- and 3′-untranslated regions (UTRs) (1, 3–5). The 5′-UTR of the D2 mRNA is ~700 nt long and has an inhibitory effect on D2 translation. It causes a fivefold reduction in D2 activity in HEK-293 cells (9). This 5′-UTR contains three short open reading frames [sORFs (1, 4, 5); Fig. 1]. The most upstream sORF (ORFa) is considered to be primarily responsible for the inhibitory effect of this 5′-UTR, since mutation of the start codon of ORFa completely abolished this inhibitory effect (9).

In this study, we scanned the 5′-UTR of D2 for the occurrence of polymorphisms and identified a single nucleotide polymorphism (SNP) in ORFa, the sORF primarily responsible for the inhibitory effect of the 5′-UTR. The association of this SNP and of the previously identified D2-Thr92Ala (19, 24) with serum iodothyronine levels and their ratios was investigated in a population of blood donors and in a population of elderly men.

MATERIALS AND METHODS

Study Populations

Healthy blood donors. Blood was collected from 158 healthy, anonymized blood donors at the Sanquin Blood Bank South West Region (Rotterdam, The Netherlands) (24). Informed consent was given by all donors. One subject was excluded because of serum free (f)T4 and TSH levels, indicating hyperthyroidism, another because of hypothyroid fT4 and TSH levels. Sex was not documented for one subject. The mean age of the study population was 46.2 ± 12.2 yr (mean ± SD); 47.4 ± 10.9 yr in the males (n = 100), and 44.6 ± 13.9 yr in the females (n = 55). No information on possible thyroid hormone treatment was available. Descriptive statistics of this population are shown in Table 1. No anthropometric data were available for this population. DNA was extracted from 2 ml of blood, using the PUREGENE DNA Isolation Kit (Genta Systems, Minneapolis, MN) with slight modifications of the provided protocol. After isolation, DNA concentration was measured at 260 nm, and all samples were diluted to a concentration of 50 ng/μl (stock) and 10 ng/μl (work solution). Purity was determined by measuring the 260- to 280-nm ratio.
Healthy elderly men. DNA was available of 349 men of a cross-sectional, single-center study in 403 independently living men, who were 70 yr of age and older (36). In this study, names and addresses of all male inhabitants of 70 yr and older were drawn from the municipal register of Zoetermeer, a medium-sized town in the western part of The Netherlands. A total of 1,567 men were invited, and, after exclusion of subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently, eventually 403 men participated (25.7%). Participants signed an informed consent. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. See Ref. 36 for a more detailed description of this population. Twenty-six subjects were not of Caucasian origin, the majority of them being of Asian origin. See Table 2 for the description of this population. Of the 349 subjects, five were receiving thyroid hormone replacement therapy.

**Serum and Plasma Analyses**

Healthy blood donors. In this population, all hormone measurements were performed in EDTA plasma. T4, rT4, T3, and TSH were measured by chemiluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Rochester, NY). Reverse (r)T3 was measured by radioimmunoassay (RIA), as previously described (37). Intra- and interassay variability coefficients of all the assays were below 11%.

Serum total cholesterol was determined enzymatically on a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN). Both high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined by a homogeneous assay (Roche Diagnostics, Indianapolis, IN). Both high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined by a homogeneous assay (Roche Diagnostics, Indianapolis, IN). HDL-C was calculated as total cholesterol minus LDL-C minus triglycerides divided by 5.

**Sequence Analysis**

The 5'-UTR of the D2 gene was analyzed for the occurrence of SNPs by sequence analysis in 15 randomly selected blood donors. First, genomic DNA was amplified by polymerase chain reaction (PCR) using the primers listed in Table 3. All primers used in this study were ordered from Invitrogen (Breda, The Netherlands). The PCR conditions were as follows: 5 min at 96°C; 38 cycles of 1 min at 94°C, 1 min at annealing temperature (Table 3), and 1 min at 72°C; and finally 7 min at 72°C. PCR products were verified by agarose gel electrophoresis. Subsequently, they were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics). To increase specificity, sequencing of the PCR products was performed using internal primers (Table 3). Samples were purified using Micro Bio-Spin P-30 Tris columns (Bio-Rad, Veenendaal, The Netherlands) and sequenced directly on an automated ABI 3100 capillary sequencer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), using the Big Dye Terminator Cycle Sequencing method (Applied Biosystems).

**Genotyping**

The genotypes of both populations were determined by single base extension (SBE) analysis as described previously (24). PCR products were generated using the primers listed in Table 3. Twenty nanograms of genomic DNA were amplified in a PCR reaction with a total volume of 10 μl. The PCR mixture contained 1× PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl2, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen). Annealing temperatures of the PCRs are listed in Table 3. The SBE reactions were performed using the ABI Prism SNaPshot ddNTP Primer Extension Kit (Applied Biosystems) with slight modifications of the protocol provided by the manufacturer. Forward primers were used for SBE analysis of both the D2-Thr92Ala and the D2-ORFa-Gly3Asp polymorphisms.

**Statistical Analysis**

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Chicago, IL). Differences between the genotype groups were adjusted for multiple testing by 10.220.33.4 on June 24, 2017 http://ajpendo.physiology.org/ Downloaded from
age and/or sex if appropriate and tested by analysis of covariance
using the general linear model procedure. Bonferroni correction for
multiple testing was used where necessary. In case of an allele dose
effect, we performed a (multiple) linear regression analysis to quantify
the association. To minimize the influence of outliers, logarithmic
transformations were performed if appropriate. The effects of the
polymorphisms on serum indexes of thyroid function in the elderly
were analyzed after exclusion of the subjects on thyroid hormone
treatment (n = 5100). Deviation from Hardy-Weinberg equilibrium was
analyzed using a χ2 test. P values are two-sided throughout, and P <
0.05 was considered significant. Haplotype allele frequencies were
estimated using the PHASE program (35).

<table>
<thead>
<tr>
<th>PCR Primers</th>
<th>TmP</th>
<th>Sequencing SBE Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fw</td>
<td>5′-TCGCCATGCTGGCCGCTGTC-3′</td>
<td>60°C</td>
</tr>
<tr>
<td>Fw</td>
<td>5′-TCGCCATGCTGGCCGCTGTC-3′</td>
<td>60°C</td>
</tr>
<tr>
<td>Fw</td>
<td>5′-TCGCCATGCTGGCCGCTGTC-3′</td>
<td>50°C</td>
</tr>
<tr>
<td>Fw</td>
<td>5′-TCGCCATGCTGGCCGCTGTC-3′</td>
<td>55°C</td>
</tr>
</tbody>
</table>

SBE analysis

D2-Thr92Ala

Fw | 5′-CAGCTATCTTCTCCTGATCAAC-3′ | 58°C | 5′-(T16)GTGTGGTGCATGTCTCCAGT-3′ |

D2-ORFa-G/A

Fw | 5′-CTGAGGAGAGACTTGCATG-3′ | 50°C | 5′-(T40)TGAGCATAGAGACAAATGAAAG-3′ |

SBE, single base extension; 5′-UTR, 5′-untranslated region; Fw, forward; Rv, reverse; D2, type II deiodinase.

Table 3. Conditions and primers used for PCR amplification and sequencing and SBE analysis of D2

![Fig. 2. Differences in plasma iodothyronine levels between subjects with 0, 1, or 2 copies of the Asp1 allele of D2-ORFa in a population of 156 blood donors. To convert thyroxine (T4) to μg/dl and FT4 to ng/dl, multiply by 0.0777; to convert triiodothyronine (T3) and reverse (r)T3 to ng/dl, multiply by 65.1. FT4, free T4.](http://ajpendo.physiology.org/)

AJP-Endocrinol Metab • VOL 289 • JULY 2005 • www.ajpendo.org
RESULTS

Identification of New SNPs

Sequencing of the 5’-UTR of D2 revealed one SNP (GGC to GAC) located in the previously described ORFa [AGG AAA GCC TAA; Fig. 1 (1)]. This variation changes the third codon for Gly into a codon for Asp. SBE analysis showed a frequency of 33.9% of the Asp3 allele in the population of blood donors for Gly into a codon for Asp. SBE analysis showed a frequency of 33.5% in the population of elderly men. Furthermore, the allele frequency of the Asp3 allele of D2-ORFa was not associated with any thyroid parameters in the population of blood donors or in the population of elderly men. Separate analysis of male and female subjects gave similar results as analysis of carriers and noncarriers. In this population, no association of the D2-ORFa-Gly3Asp and/or D2-Thr92Ala variant was observed with serum TSH, iodothyronine levels, or their ratios (Table 4). Association analysis of subjects with 0, 1, or 2 copies of the risk allele gave similar results as analysis of carriers and noncarriers.

Association of D2-ORFa-Gly3Asp with Circulating TSH and Iodothyronine Levels

Blood donors. The Asp3 allele of D2-ORFa was associated with decreasing levels of T4, rT3, and T3 in a dose-dependent manner: Gly/Gly 90.82 ± 1.82, Gly/Asp 86.78 ± 1.87, and Asp/Asp 80.96 ± 3.48 nmol/l for T4 (P = 0.01, mean ± SE, corresponding to 7.06 ± 0.14, 6.74 ± 0.15, and 6.29 ± 0.27 μg/dl; Fig. 2A); and Gly/Gly 15.67 ± 0.28, Gly/Asp 14.86 ± 0.28, and Asp/Asp 13.65 ± 0.53 pmol/l for rT3 (P = 0.001, corresponding to 1.22 ± 0.02, 1.16 ± 0.02, and 1.06 ± 0.04 ng/dl; Fig. 2B); and Gly/Gly 0.33 ± 0.01, Gly/Asp 0.31 ± 0.01, and Asp/Asp 0.28 ± 0.02 nmol/l for rT3 (P = 0.01, corresponding to 21 ± 1, 20 ± 1, 18 ± 1 ng/dl; Fig. 2D). No effect of the Asp3 allele was observed on plasma T3 levels (P = 0.59; Fig. 2C) and circulating TSH levels (P = 0.54).

Furthermore, the Asp3 allele was associated with an increasing T3/T4 ratio (P = 0.002) and T3/rT3 ratio (P = 0.03), which are both thought to better reflect tissue deiodinase activity (Fig. 3). The difference in T3/T4 ratio corresponds to a 0.36 SD increase per allele copy.

Separate analysis of male and female subjects gave similar associations of the Asp3 allele with iodothyronine levels in both groups, although it failed to reach significance in the females (n = 55).

The D2-Thr92Ala polymorphism was not associated with plasma iodothyronines in this population, as published previously (24).

Healthy elderly men. Due to limited quality and quantity of the DNA, only 341 subjects could be genotyped for both SNPs. In this population, no association of the D2-ORFa-Gly3Asp and/or D2-Thr92Ala variant was observed with serum TSH, iodothyronine levels, or their ratios (Table 4). Association analysis of subjects with 0, 1, or 2 copies of the risk allele gave similar results as analysis of carriers and noncarriers.

Association of D2-ORFa-Gly3Asp with Plasma Cholesterol Levels

Blood donors. The D2-ORFa-related differences in serum thyroid parameters were not accompanied by alterations in cholesterol metabolism, since the D2-ORFa-Asp3 variant was not associated with serum cholesterol, HDL-C, or LDL-C in this population (Table 5). The D2-Thr92Ala polymorphism was also not associated with plasma cholesterol levels (data not shown). It should be noted, however, that plasma T4 and T3 were not correlated with plasma cholesterol levels in this population either.

Table 4. Plasma TSH and iodothyronine levels in carriers of the D2-Thr92Ala and in carriers of the D2-ORFa-Gly3Asp variant in a population of elderly men

<table>
<thead>
<tr>
<th></th>
<th>Thr/Thr</th>
<th>Thr/Ala + Ala/Ala</th>
<th>P</th>
<th>Gly/Gly</th>
<th>Gly/Gly + Asp/Asp</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>134</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH, mU/l</td>
<td>1.08±0.08</td>
<td>1.19±0.07</td>
<td>0.64*</td>
<td>1.19±0.07</td>
<td>1.20±0.08</td>
<td>0.99</td>
</tr>
<tr>
<td>T4, nmol/l</td>
<td>80.42±1.28</td>
<td>81.35±1.05a</td>
<td>0.57</td>
<td>81.00±1.22b</td>
<td>81.00±1.11</td>
<td>0.99</td>
</tr>
<tr>
<td>rT3, pmol/l</td>
<td>16.82±0.27</td>
<td>16.51±0.22</td>
<td>0.38</td>
<td>16.67±0.26</td>
<td>16.62±0.23</td>
<td>0.87</td>
</tr>
<tr>
<td>T3, nmol/l</td>
<td>1.42±0.02</td>
<td>1.45±0.02</td>
<td>0.31</td>
<td>1.45±0.02</td>
<td>1.43±0.02</td>
<td>0.44</td>
</tr>
<tr>
<td>rT3, nmol/l</td>
<td>0.34±0.01</td>
<td>0.33±0.01</td>
<td>0.50</td>
<td>0.33±0.01</td>
<td>0.33±0.01</td>
<td>0.90</td>
</tr>
<tr>
<td>T3/rT3</td>
<td>1.82±0.03</td>
<td>1.83±0.03a</td>
<td>0.75</td>
<td>1.84±0.03b</td>
<td>1.81±0.03</td>
<td>0.51</td>
</tr>
<tr>
<td>T4/T3</td>
<td>4.56±0.13</td>
<td>4.74±0.11</td>
<td>0.28</td>
<td>4.71±0.12</td>
<td>4.62±0.11</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Data represent means ± SE; *n = 199, b n = 147. *P value for deviation from Hardy-Weinberg equilibrium.
Table 5. Plasma cholesterol levels in carriers and noncarriers of the D2-ORFa-Gly3Asp variant in healthy blood donors

<table>
<thead>
<tr>
<th></th>
<th>D2-ORFa Gly/Gly</th>
<th>D2-ORFa Gly/Asp + Asp/Asp</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>67</td>
<td>82</td>
<td>0.70*</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>5.65±0.12</td>
<td>5.42±0.11</td>
<td>0.17</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>1.35±0.04</td>
<td>1.30±0.04</td>
<td>0.43</td>
</tr>
<tr>
<td>LDL-C, mmol/l</td>
<td>3.69±0.11</td>
<td>3.55±0.10</td>
<td>0.34</td>
</tr>
<tr>
<td>HDL-C/LDL-C ratio</td>
<td>0.40±0.02</td>
<td>0.41±0.02</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Data represent means ± SE, data are adjusted for age and sex. HDL-C and LDL-C, high- and low-density lipoprotein-cholesterol, respectively. *P value for deviation from Hardy-Weinberg equilibrium.

Haplotype Analysis of D2-Thr92Ala and D2-ORFa-Gly3Asp

D2-Thr92Ala and D2-ORFa-Gly3Asp were analyzed for linkage disequilibrium in both populations, and haplotype allele frequencies were estimated. Frequency estimates of the four different haplotype alleles are shown in Fig. 4. The haplotype allele D2-ORFa-Asp³/Ala² (haplotype allele 4) was present only at very low frequency (4 copies in the blood donors and 8 in the elderly men), suggesting that the D2-ORFa-Asp³ and D2-Ala² alleles appear predominantly on different haplotype alleles and are in linkage disequilibrium (P < 0.001 for both populations). Because of the low frequency of the haplotype allele 4, the variant alleles are assigned only to frequent haplotypes (the Asp³ allele to haplotype 2 and the Ala² allele to haplotype 3). Association analysis of haplotype allele 2 with serum thyroid parameters in blood donors, therefore, gave similar results to the association of the D2-ORFa-Asp³ variant with these parameters.

DISCUSSION

In this study, we identified a new SNP in the most upstream sORF (ORFa) of the 5'-UTR of D2. ORFa contains only three codons and a stop codon (Met-Lys-Gly) and is primarily responsible for the inhibitory effect of this 5'-UTR on D2 translation (9). The polymorphism identified in this study results in a change of the last codon from a Gly to an Asp. In healthy blood donors, this D2-ORFa-Asp³ variant was associated with lower levels of plasma T₄, rT₄, and rT₃ in a dose-dependent manner, but not with plasma T₃ and TSH levels. Pituitary D2 activity is essential for the negative feedback regulation of hypophysal TSH secretion by circulating T₄ (32, 34). D2 knockout mice have an impaired feedback regulation, resulting in an approximately twofold-increased T₄ and TSH concentration but normal circulating T₃ (32). The relatively low T₄ but unaltered TSH and T₃ levels in carriers of the D2-ORFa-Asp³ variant suggest that the pituitary needs less T₄ to produce the same amounts of local T₃ and, thus, a normal negative feedback action. These data therefore suggest that the D2-ORFa-Asp³ variant results in a higher D2 activity in the pituitary. As we described previously (24), the D2-Thr92Ala polymorphism was not associated with plasma iodothyronines in this population.

D2 has been identified in skeletal muscle of humans and pigs, especially in the hypothyroid situation (10, 30, 38), although we were not able to measure D2 activity in skeletal muscle of critically ill patients (25). On the basis of these observations and on the fact that propylthiouracil, an inhibitor of D1 but not of D2, can only partially inhibit T₄-to-T₃ conversion in T₄-replaced hypothyroid subjects (8, 16, 29), it is believed that D2 is not only involved in the regulation of local thyroid hormone bioactivity but that it also contributes to circulating iodothyronine levels (2). Plasma iodothyronine levels depend not only on the iodothyronine-metabolizing enzymes but also, among other things, on thyroid function and plasma iodothyronine binding capacity. Therefore, the ratios between plasma iodothyronines are thought to better reflect peripheral metabolism of thyroid hormone. The association of the D2-ORFa-Asp³ variant with higher T₃/T₄ and T₃/rT₃ ratios supports the hypothesis that D2 contributes to circulating iodothyronine levels. Higher T₃/T₄ and T₃/rT₃ ratios in carriers of the D2-ORFa-Asp³ variant suggest that these subjects have a higher D2 activity in tissues such as skeletal muscle and that this altered D2 activity indeed affects plasma iodothyronine levels. The low serum rT₃ levels in carriers of the D2-ORFa-Asp³ variant support this, as D2 can also break down rT₃ by outer-ring deiodination (2). The D2-ORFa-related differences in plasma thyroid parameters were not accompanied by alterations in cholesterol metabolism, since the D2-ORFa-Asp³ variant was not associated with plasma cholesterol, HDL-C, or LDL-C in this population. However, it should be noted that T₄ and T₃ were not correlated with serum cholesterol levels in this population either.

Only 10% of all mRNA sequences contain a sORF upstream of the major coding sequence (12, 13). Another example of such a gene is the β₂-adrenergic receptor, whose 5'-UTR contains a sORF, consisting of 19 codons and a stop codon, which inhibits the translation of the receptor protein (22). A polymorphism in this sORF, which alters the last codon from an Arg to a Cys, results in a twofold increase in the translational efficiency of the β₂-adrenergic receptor (18). Similarly, the D2-ORFa-Gly3Asp polymorphism may very well result in an increased translational efficiency of D2.

![Fig. 4. Four haplotype alleles that were constructed from the D2-ORFa-Gly3Asp and the D2-Thr92Ala polymorphisms. Frequency estimates of the different haplotype alleles in the 2 different populations are shown.](image-url)
There are several other potential mechanisms through which the codon change in ORFa might modulate D2 activity. The polymorphism is located closely to a potential intron, which is spliced out alternatively, producing variant 2 of human D2 mRNA. The D2-ORFa-Gly3Asp polymorphism may thus affect alternative splicing of this intron. Furthermore, because this SNP is located in the 5′-UTR, a change in the stability of the mRNA or in secondary structure may also play a role in the inhibitory effect of 5′-UTR sORFs on translational efficiency (11). Alternatively, the D2-ORFa-Gly3Asp polymorphism could be in linkage disequilibrium with another SNP located in the coding sequence, such as D2-Thr92Ala, or in a regulatory area of the gene. However, in this study, haplotype analysis indicated that the risk alleles appear largely on separate (frequent) haplotype alleles. The D2-ORFa-Gly3Asp polymorphism does not seem to be related to insulin resistance (unpublished data), in contrast to D2-Thr92Ala, which is associated with insulin resistance (19). Vice versa, D2-Thr92Ala was not related to thyroid hormone levels.

In a population of elderly men, the D2-ORFa-Gly3Asp variant was not associated with plasma iodothyronine levels. The different effects of the D2-ORFa-Asp3 allele in blood donors and elderly men might be explained by the difference in age between the two populations (mean ages 46 vs. 77 yr). Throughout adult life, skeletal muscle size and strength gradually decline, resulting in a decrease in D2-expressing skeletal muscle. Although D1 activity also decreases during aging (7, 21), the relative contribution of D2 to serum iodothyronine production may be less important in elderly than in young subjects (23). The association of the D2-ORFa-Gly3Asp variant with plasma iodothyronine levels in blood donors was strongest in the youngest age quartile, whereas this association failed to reach significance in the oldest age quartile, which supports this hypothesis (data not shown).

In conclusion, we identified a polymorphism in ORFα of the 5′-UTR of D2, which is the sORF primarily responsible for the inhibitory effect of the 5′-UTR on D2 expression. This D2-ORFa-Gly3Asp polymorphism is associated with circulating iodothyronine levels in blood donors but not in elderly men, suggesting an age-dependent effect of this polymorphism.

ACKNOWLEDGMENTS

We thank Ronald van de Wal for assistance with the direct sequencing, Wendy Hugen for help with DNA isolation, and Pascal Arp for help with the SNPshot analyses.

GRANTS

This work was supported by ZonMw Grant 920-03-146 (R. P. Peeters).

REFERENCES


