Polymorphisms in Thyroid Hormone Pathway Genes Are Associated with Plasma TSH and Iodothyronine Levels in Healthy Subjects

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Single nucleotide polymorphisms (SNPs) in genes involved in thyroid hormone metabolism may affect thyroid hormone bioactivity. We investigated the occurrence and possible effects of SNPs in the deiodinases (D1–D3), the TSH receptor (TSHR), and the T3 receptor β (TRβ) genes.

SNPs were identified in public databases or by sequencing of genomic DNA from 15 randomly selected subjects (30 alleles). Genotypes for the identified SNPs were determined in 156 healthy blood donors and related to plasma T4, free T4, rT3, and TSH levels.

Eight SNPs of interest were identified, four of which had not yet been published. Three are located in the 3′-untranslated region: D1a-C/T (allele frequencies, C = 66%, T = 34%), D1b-A/G (A = 89.7%, G = 10.3%), and D3-7/TG (T = 85.5%, G = 14.2%). Four are missense SNPs: D2-A/G (Thr92Ala, Thr = 62.2%, Ala = 38.8%), TSHRa-G/C (Asp36His, Asp = 99.4%, His = 0.6%), TSHRb-C/A (Pro52Thr, Pro = 94.2%, Thr = 5.8%), TSHRc-C/G (Asp727Glu, Asp = 90.7%, Glu = 9.3%). One is a silent SNP: TRβ-T/T (T = 96.8%, C = 3.2%). D1a-T was associated in a dose-dependent manner with a higher plasma rT3 [CC, 0.29 ± 0.01; CT, 0.32 ± 0.01; and TT, 0.34 ± 0.02 nmol/liter (mean ± SE); P = 0.017], a higher plasma rT3/T3 (P = 0.01), and a lower T3/rT3 (P = 0.003) ratio. The D1b-G allele was associated with lower plasma rT3/T3 (P = 0.024) and with higher T3/rT3 (P = 0.08) ratios. TSHRc-G was associated with a lower plasma TSH (CC, 1.38 ± 0.07 vs. GC, 1.06 ± 0.14 mU/liter; P = 0.04), and with lower plasma TSH/free T4 (P = 0.06), TSH/T3 (P = 0.06), and TSH/T4 (P = 0.08) ratios. No associations with TSH and iodothyronine levels were found for the other SNPs.

We have analyzed eight SNPs in five thyroid hormone pathway genes and found significant associations of three SNPs in two genes (D1, TSHR) with plasma TSH or iodothyronine levels in a normal population. (J Clin Endocrinol Metab 88: 2880–2888, 2003)

THYROID HORMONES PLAY an essential role in a variety of metabolic and developmental processes in the human body. Most effects are mediated by the active thyroid hormone T3 via a mechanism of T3-regulated gene expression (1). Examples are the effects of thyroid hormone on brain development and skeletal maturation, on the secretion and metabolic turnover of different hormones, and on the contractility of the heart (2–6). Production of thyroid hormone, in particular the prohormone T4, is controlled by the classic hypothalamic-pituitary-thyroid axis, whereas the biological activity of thyroid hormone T3, is largely regulated by the iodothyronine deiodinases D1, D2, and D3 (4, 7, 8).

Polymorphisms (variations in the nucleotide sequences of the genome that occur in at least 1% of the population) in one or more genes involved in thyroid hormone metabolism may have subtle effects on thyroid hormone levels and thyroid hormone bioactivity throughout life. The symptoms of subclinical hyper- and hypothyroidism show that minor changes in thyroid hormone levels can have important consequences for quality of life, cognition, cholesterol metabolism, heart rate, bone mineral density, and atherosclerosis (9–14). For this reason, we investigated genes encoding key proteins in thyroid hormone metabolism for the occurrence of single nucleotide polymorphisms (SNPs). The genes selected include the three selenodeiodinase genes D1, D2, and D3, the genes for the TSH receptor (TSHR), and the T3 receptor β (TRβ). These genes were screened for SNPs, preferentially those located in exons, by searching the public SNP database (dbSNP), the human expressed sequence tag database (dbEST), as well as the literature.

D1, D2, and D3 contain a selenocysteine (Sec) in their catalytic center, encoded by a UGA codon (15–17). In the vast majority of mRNAs, UGA is recognized as a stop codon. The incorporation of Sec into the deiodinases requires the presence of a Sec insertion sequence (SECIS) element in the 3′-untranslated region (UTR) of the mRNA and several trans-acting factors (18). Because a SNP in this region may have important consequences for the production of functional deiodinase, the D1, D2, and D3 SECIS elements were sequenced in 15 randomly selected healthy subjects. Because little information is available in the databases about the possible polymorphisms in human D3, we also decided to analyze the coding sequence of the human D3 gene in these 15 subjects. A population of 156 healthy blood donors was genotyped with regard to these SNPs, and for each polymorphism
the genotype was correlated with a specific set of plasma thyroid indexes.

For the SNPs in the three deiodinases, we analyzed correlations with plasma T₄, T₃, and rT₃ and with iodothyronine ratios. Plasma iodothyronine levels depend not only on the activities of iodothyronine-metabolizing enzymes but also, among other things, on thyroid function and plasma iodothyronine-binding capacity. Therefore, ratios between plasma iodothyronines are thought to better reflect tissue deiodinase activities. Local D₂ activity is essential for the negative feedback regulation of hypothalamic TSH secretion by plasma T₄, as demonstrated in the D₂ knockout mouse (19). Therefore, the relation between the D₂ SNP and plasma TSH, and TSH/T₄ and TSH/free T₄ (FT₄) ratios was also analyzed. For the SNPs in the TSHR and in TRβ, we analyzed the correlation with plasma iodothyronines and TSH levels, and with the ratios thereof.

**Subjects and Methods**

**Study population**

Blood was collected from 158 healthy anonymized blood donors at the Sanquin Blood Bank South West region (Rotterdam, The Netherlands). Informed consent was given by all donors. One subject was excluded because of plasma FT₄ and TSH levels indicating hyperthyroidism, another because of hypothyroid FT₄ and TSH levels. Gender was not documented for one subject. The study population thus consisted of 100 males and 55 females with an average age of 46.2 yr (mean ± sd); 47.4 ± 10.9 yr in the males, and 44.6 ± 13.9 yr in the females. Donors on thyroid hormone treatment are not excluded from donation. Although ethnic background of donors has not been documented, over 99% may be of Caucasian origin. Descriptive statistics of this population are shown in Table 1.

**Table 1.** Descriptive statistics of the population analyzed in this study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± sd</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>46.3 ± 12.2'</td>
<td>4.3</td>
</tr>
<tr>
<td>TSH (mU/liter)</td>
<td>1.32 ± 0.75'</td>
<td>0.4</td>
</tr>
<tr>
<td>T₄ (nmol/liter)</td>
<td>87.96 ± 15.9</td>
<td>58-128</td>
</tr>
<tr>
<td>FT₄ (pmol/liter)</td>
<td>15.06 ± 2.4</td>
<td>11-25</td>
</tr>
<tr>
<td>rT₃ (nmol/liter)</td>
<td>0.196 ± 0.24</td>
<td>1.43-5.61</td>
</tr>
</tbody>
</table>

' TSH and iodothyronine levels of this population were determined in plasma. *Reference values used in our lab, with TSH and iodothyronine levels of this population were determined as follows: 5 min at 96 C; 35 cycles of 1 min at 94 C, 1 min at annealing temperature (Table 2), 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 PureGene PCR Purification Kit (Roche Diagnostics, Almere, The Netherlands). To increase specificity, sequencing of the PCR products was performed using internal primers (Table 2). Samples were purified using Micro Bio-Spin P-30 Tris columns (Bio-Rad Laboratories, Inc., Venennaal, The Netherlands), and sequenced directly on an automated ABI 310 capillary sequencer (Applied Biosystems), using the Big Dye Terminator Cycle Sequencing method (Applied Biosystems).

**Database searches**

Nucleotide sequences of the genes of interest were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nim.nih.gov). The TBLASTN program was used to search the dbEST for sequences that differ in one or more nucleotides from the wild-type (WT) gene. To exclude sequencing artifacts, at least two ESTs with the same nucleotide difference were required before this variation was marked as a potential SNP. In addition, the SNP database of NCBI (dbSNP) was searched.

**Restriction fragment length polymorphism (RFLP) analysis**

PCR-RFLP procedures were developed for the D1α-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRa-Asp/His, TSHRb-Pro/Thr, and TSHRc-Thr/Ala polymorphisms. The primers used are listed in Table 3. For D2-A/G, a mismatch primer was used to destroy a second restriction site for RsaI. For D3-T/G and TSHRb-C/A, mismatch primers were generated to destroy restriction sites for NsiI and SphI, respectively. The RFLP for TSHRb-C/A was described previously (21). Twenty nanograms of genomic DNA were amplified in a PCR with a total volume of 10 μl. The PCR mixture was amplified in 1× PCR buffer (Invitrogen), 0.2 mm of each

**Table 2.** Conditions used for PCR amplification and subsequent sequencing of gDNA regions of interest

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR</th>
<th>Annealing temperature</th>
<th>Sequencing PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>SECIS-D1 Fw</td>
<td>5'-GTGAAATGCTTCCACTAGTCA-3'</td>
<td>50C</td>
<td>5'-CAACACAGATCTACTAAGGAGGA-3'</td>
</tr>
<tr>
<td>SECIS-D2 Fw</td>
<td>5'-CTTCTCAGGAGCCACACAG-3'</td>
<td>50C</td>
<td>5'-CCTCGAGGAGGGCGAGAGG-3'</td>
</tr>
<tr>
<td>SECIS-D3 Fw</td>
<td>5'-ATGCTTTGGCCGGTGCTTC-3'</td>
<td>50C</td>
<td>5'-GCGGTAGGAGGGCCGGTGG-3'</td>
</tr>
<tr>
<td>SECIS-D1 Rv</td>
<td>5'-CCCGAGGTGGTTCTGCCAG-3'</td>
<td>50C</td>
<td>5'-GCGGTAGGAGGGCCGGTGG-3'</td>
</tr>
<tr>
<td>SECIS-D2 Rv</td>
<td>5'-CCGGCTCACTGGCCGCATGA-3'</td>
<td>50C</td>
<td>5'-GCGGTAGGAGGGCCGGTGG-3'</td>
</tr>
<tr>
<td>SECIS-D3 Rv</td>
<td>5'-AGTCCAGCAGAGGGCCAC-3'</td>
<td>50C</td>
<td>5'-GCGGTAGGAGGGCCGGTGG-3'</td>
</tr>
</tbody>
</table>
deoxynucleoside triphosphate, 1.5 mm MgCl₂, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen). Annealing temperatures of the different PCRs are listed in Table 3. Five units of restriction enzyme were used for a 1-h digestion of the PCR product at the recommended temperature. Table 3 lists the restriction enzymes used for RFLP analysis of the different SNPs. Digestion products were analyzed by agarose gel electrophoresis. All subjects were genotyped for the D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-C/G, and TRβ-T/C polymorphisms using RFLP and SBE analysis (see Methods for details).

**Functional characterization of D2-Thr92Ala variant**

A human D2 expression vector (pcDNA3-hD2-rSECIS), containing the human D2 cDNA inserted 5' to the SEClS element of the rat D1 gene, was used as template for site-directed mutagenesis via the circular mutagenesis reaction (25). Overlapping sense and antisense primers containing the nucleotide changes needed to produce the Thr92Ala D2 mutant (sense 5'-TGCCTGTTGAGAATGCTCCTGGAGGATGTTGTTGAGAGAATAGC-3', antisense 5'-ATATTTTCATCATTCTGTTAATATTTTCATCATTCTGTTA-3') were used. The mutant D2-Thr92Ala cDNA was sequenced directly on an automated ABI Prism 377 DNA Sequencer (Applied Biosystems) with slight modifications of the protocol provided by the manufacturer. For SBE analysis of the D1a, D3, and TRß alleles, the reverse primers were used, and for the other alleles, the forward primers were used.

The principle of the in vitro D2 activity assay is the production of radiodiode by outer-ring deiodination of [3,5'125I]T3, or [3,5'-125I]T4. Duplicate incubations contained about 100,000 cpm labeled T4 or T3, with varying amounts of unlabeled substrate (T4 or T3) and COS cell homogenates (50–100 μg protein) in a total volume of 0.2 ml of TED20 buffer [20 mM dithiothreitol (DTT)]. Mixtures were incubated for 60 min at 37 °C, whereafter the production of radiodiode was determined as described (22).

The principle of the in situ D2 activity assay is the release of [125I]T3 in the medium of D2 expressing COS-1 cells incubated with [3,5'-125I]T4. COS cells were cultured in 6-well plates and transfected with 2.5 μg of plasmid per well as described (22). One day after transfection, cell monolayers were washed with serum-free DMEM/F12 medium and then cultured for an additional 24 h in serum-free DMEM/F12 (plus 40 nm selenite), to which were added [125I]T4 (106 cpm/ml) and 1 μM unlabeled T4. Medium was harvested, extracted, and analyzed by HPLC as described (22).

**Statistical analysis**

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL). Logarithmic transformations were applied to normalize variables and to minimize the influence of outliers if applicable. Differences in plasma thyroid hormone levels between the genotype groups were adjusted for age and sex and tested by analysis of covariance using the general linear model procedure. Results are reported as mean ± se in the figures, and as mean ± se in the tables. Comparison of the frequencies of the genotypes between males and females was carried out using a χ² test. Deviation from Hardy-Weinberg equilibrium was also analyzed using a χ² test. P values are two-sided throughout, and P less than 0.05 was considered significant. Haploftype allele frequencies were estimated using the computer program 3LOCUS.pas (24).

**Results**

**Identification of SNPs**

Figure 1 shows an overview of the different SNPs identified. Previous unpublished studies in our laboratory had shown a variation at nucleotide position 785 of the D1 cDNA sequence (GenBank accession no. GI 4557521) which is occupied by a C or a T. This polymorphism was confirmed in the dbEST and is referred to as D1a-C/T (Fig. 1). Sequencing of the region around the SEClS element of the D1 gene showed an A/G variation in 2 of the 15 subjects at position 1814 (D1b-A/G); 33 nucleotides downstream of the SEClS element. This variation was confirmed in the dbEST. No

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**Table 3. Conditions used for RFLP and SBE analysis (see Subjects and Methods)**

<table>
<thead>
<tr>
<th>RFLP primers</th>
<th>Annealing temperature</th>
<th>Restriction enzymes</th>
<th>SBE primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1a-C/T</td>
<td>54°C</td>
<td>BclI</td>
<td>Fw 5'- (T4) GTAACCTGAATTTCTAAGTG-3', Rv 5'- (T4) GATGAGAAGCCCTCCGGTGG-3'</td>
</tr>
<tr>
<td>D1b-A/G</td>
<td>48°C</td>
<td>SpeI</td>
<td>Fw 5'- (T4) TTAAGAGTACGAAACTA-3', Rv 5'- (T4) ATATTTGATCATCATTGTA-3'</td>
</tr>
<tr>
<td>D2-A/G</td>
<td>58°C</td>
<td>Rsal</td>
<td>Fw 5'- (T4) GTTGGTGCTGTTGCTCAAGT-3', Rv 5'- (T4) AGCTGTTGCTTCCCTCCTG-3'</td>
</tr>
<tr>
<td>D3-C/G</td>
<td>60°C</td>
<td>BsaSI</td>
<td>Fw 5'- (T4) GCTGAGGAAAGGATACA-3', Rv 5'- (T4) GTTGGAGAAGGATGTA-3'</td>
</tr>
<tr>
<td>TSHa-D2</td>
<td>53C</td>
<td>Bsp 1286I</td>
<td>Fw 5'- (T4) GCTGGTGCTGTTGCTCAAGT-3', Rv 5'- (T4) GTTGGAGAAGGATGTA-3'</td>
</tr>
<tr>
<td>TSHb/C/A</td>
<td>58C</td>
<td>Thh111</td>
<td>Fw 5'- (T4) AAGCGTCGCCGGTCCATTGC-3', Rv 5'- (T4) CACTACCGACCTGCACTG-3'</td>
</tr>
<tr>
<td>TShc/C/G</td>
<td>60C</td>
<td>NlaI</td>
<td>Fw 5'- (T4) GTTACAAAGGTTACACCACGA-3', Rv 5'- (T4) TGTGGAGAAGGATGTA-3'</td>
</tr>
<tr>
<td>TRβ-T/C</td>
<td>56C</td>
<td>AatII</td>
<td>Fw 5'- (T4) AAGAAAGGAGCTGCACTATC-3', Rv 5'- (T4) GCCTCCGACCTGCACTATC-3'</td>
</tr>
</tbody>
</table>

Mismatches are shown by an underscore.

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**Table 9. Identification of SNPs**

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Description</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1a-C/T</td>
<td>D1a-C/T</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>D1b-A/G</td>
<td>D1b-A/G</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>D2-A/G</td>
<td>D2-A/G</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>D3-C/G</td>
<td>D3-C/G</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>TSHa-D2</td>
<td>TSHa-D2</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td>TSHb/C/A</td>
<td>TSHb/C/A</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>TShc/C/G</td>
<td>TShc/C/G</td>
<td>0.67 ± 0.02</td>
</tr>
<tr>
<td>TRβ-T/C</td>
<td>TRβ-T/C</td>
<td>0.33 ± 0.01</td>
</tr>
</tbody>
</table>
other SNPs in D1 were found in at least two different databases.

No variation was detected by sequencing of the region around the SECIS element of the D2 gene. However, screening of the dbEST showed a very frequent A/G polymorphism at nucleotide 674 of the D2 sequence (GI 13654872), predicting a change in amino acid 92 of the protein (D2-G/A; Thr92Ala; Fig. 1).

Sequencing of the coding sequence of the D3 gene showed no variation in the 15 healthy subjects. However, a T/G polymorphism was found at position 1546 of the D3 cDNA sequence (GI 4503334), 66 nucleotides upstream of the SECIS element (D3-T/G) in 2 of the 15 subjects. This polymorphism was confirmed in the dbSNP.

Screening of the literature revealed three SNPs in the coding sequence of the TSHR gene. The Asp36His variation [TSHRa-G/C polymorphism in nucleotide 206 of the TSHR cDNA sequence (GI 4507700)] and the Pro52Thr variation (TSHRb-C/A polymorphism in nucleotide 254) are located in the extracellular domain of the receptor, whereas the Asp727Glu variation (TSHRc-C/G polymorphism in nucleotide 2281) is situated in the intracellular domain. TSHRb-C/A and TSHRc-C/G were also identified in the dbSNP.

A silent T/C polymorphism in TRβ/H9252 gene (TRβ/H9252-T/C), corresponding to position 1536 of the TRβ1 cDNA sequence (GI 10835122) and to amino acid 412 (Phe) of the TRβ1 protein, was found in previous studies in our laboratory (Wassen, F., unpublished results).

Figure 2 shows examples of one RFLP analysis and two SBE analyses. All SBE and RFLP data were concordant, with the restriction that we could not make a clear distinction by RFLP between heterozygous D3-GT and homozygous D3-GG subjects. Frequencies of the different genotypes and alleles are depicted in Table 4. Except for TSHRa-Asp36His, all SNPs were frequent enough to perform statistical analysis. Except for TSHRb-Pro52Thr, all distributions were in Hardy-Weinberg equilibrium (Table 4).

**Association of SNPs with plasma TSH and iodothyronine levels**

D1a is a very frequent polymorphism. The T-allele of D1a was associated in a dose-dependent manner with increasing plasma rT3 levels: CC, 0.29 ± 0.01; CT, 0.32 ± 0.01; and TT, 0.34 ± 0.02 nmol/liter (mean ± se; P = 0.017; corresponding to 18.8, 20.8, and 22.1 ng/dl; Fig. 3A). The difference corresponds to a 33% of sd increase per allele copy. In addition, we observed...
the T-allele to be associated with increasing plasma rT3/T4 ratio (P = 0.01; Fig. 3B) and decreasing plasma T3/rT3 ratio (P = 0.003). The G allele of the D1b-A/G polymorphism was less frequent and was associated with lower plasma rT3/T4 (P = 0.024) and higher T3/rT3 (P = 0.08) ratios. Due to the low frequency of the polymorphism, the difference in rT3 levels failed to reach significance (P = 0.17; Fig. 3, C and D).

These two D1 SNPs were analyzed for linkage disequi-

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**TABLE 4.** Frequencies of genotypes and alleles for the polymorphisms identified

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype frequencies</th>
<th>Allele frequency</th>
<th>HWE P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1a-C/T</td>
<td>CC 65 (41.7%) CT 76 (48.7%) TT 15 (9.6%)</td>
<td>C 66% T 34%</td>
<td>0.28</td>
</tr>
<tr>
<td>D1b-A/G</td>
<td>AA 124 (79.5%) AG 32 (20.5%) GG 0</td>
<td>A 89.7% G 10.3%</td>
<td>0.15</td>
</tr>
<tr>
<td>D2-A/G</td>
<td>AA 58 (37.2%) AG 75 (48.1%) GG 23 (14.7%)</td>
<td>A 61.2% G 38.8%</td>
<td>0.88</td>
</tr>
<tr>
<td>D3-T/G</td>
<td>TT 112 (72.3%) TG 42 (27.1%) GG 1 (0.6%)</td>
<td>T 85.5% G 14.2%</td>
<td>0.16</td>
</tr>
<tr>
<td>TSHa-G/C</td>
<td>GG 154 (98.7%) GC 2 (1.3%) CC 0</td>
<td>G 99.4% C 0.6%</td>
<td>0.94</td>
</tr>
<tr>
<td>TSHb-C/A</td>
<td>CC 139 (89.7%) CA 14 (9.0%) AA 2 (1.3%)</td>
<td>C 94.2% A 5.8%</td>
<td>0.03</td>
</tr>
<tr>
<td>TSHc-C/G</td>
<td>CC 127 (81.4%) CG 29 (18.6%) GG 0</td>
<td>C 90.7% G 9.3%</td>
<td>0.20</td>
</tr>
<tr>
<td>TRβ-T/C</td>
<td>TT 145 (93.5%) TC 10 (6.5%) CC 0</td>
<td>T 96.8% C 3.2%</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Except for TSHb-C/A, distribution of all genotypes was in Hardy-Weinberg equilibrium (HWE).

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**Fig. 3.** Differences between plasma rT3 concentrations (A and C) and plasma rT3/T4 ratios (B and D) by genotype for the D1a (A and B) and D1b (C and D) polymorphism. P values represent an ANOVA test (C) or an ANOVA test for linearity (A and B). To convert values for rT3 to nanograms per deciliter, multiply by 65.1.
librium, and haplotype allele frequencies were calculated. The results revealed that there were only three different haplotype alleles in this population (1 = aC-bA, 2 = aT-bA, 3 = aC-bG, with a frequency of 0.56, 0.34, and 0.10, respectively). On the basis of these three haplotype alleles, all individuals could be genotyped, and the effect of the individual alleles on plasma thyroid hormone parameters could be assessed. The haplotype 1 showed no correlation with rT3, rT3/T4, or with T3/rT3 ratio. The haplotype 2 showed a positive correlation with rT3 (P = 0.017) and rT3/T4 ratio (P = 0.01), and a negative relation with T3/rT3 ratio (P = 0.003; Fig. 4, A and B). Due to the low frequency of the haplotype allele 3, statistical analysis had limited power. A negative relation with rT3 (P = 0.172) and with rT3/T4 (P = 0.024) and a positive relation with T3/rT3 (P = 0.08) was observed.

D2-A/G was the most frequent polymorphism in this study. No significant correlation was found with plasma iodothyronine levels in this population. However, plasma TSH levels were lower in subjects heterozygous for this D2 polymorphism (GA, 1.17 ± 0.08, vs. AA, 1.44 ± 0.11, and GG, 1.49 ± 0.13 mU/liter; P = 0.07). Also, the plasma TSH/T4 ratio was lower in heterozygous subjects (P = 0.08).

For the D3-T/G polymorphism, the frequency of genotypes was 72.3% TT, 27.1% TG, and 0.6% GG. No significant correlation between this polymorphism and plasma iodothyronines was observed.

Only two subjects in this study possessed the TSHRa-His variant. Therefore, statistical power was limited to analyze the relation with plasma levels for this polymorphism. No associations were found for the TSHRb-Pro52Thr polymorphism with plasma iodothyronine or TSH levels in this population. For the TSHRc-Asp727Glu polymorphism, heterozygous subjects showed decreased TSH levels (P = 0.04; Fig. 5) and tended to have lower plasma TSH/FT4 (P = 0.06), TSH/T4 (P = 0.08), and TSH/T3 (P = 0.06) ratios than subjects homozygous for the Asp allele.

No association was found for the TRα-T/C polymorphism with plasma iodothyronine and TSH levels in our population.

**Functional characterization of D2-Thr92Ala variant**

To define the effects of the substitution of the Thr residue at position 92 in D2 by Ala, expression vectors were made encoding WT-D2 and D2-Thr92Ala enzyme. Homogenates of cells, transfected with WT-D2, D2-Thr92Ala, or a 1:1 mixture of WT-D2 and D2-Thr92Ala enzymes, displayed similar Km and Vmax values using T4 or rT3 as substrate (Table 5 and Fig. 6A). Upon incubation of intact transfected cells with increasing amounts of unlabeled T4, the fractional deiodination of [125I]T4 decreased in a similar way (Fig. 6B). No differences in deiodination of T4 were observed between cells expressing WT-D2, D2-Thr92Ala, or a combination of both.

**Discussion**

To date, little information has been available regarding the presence of functionally relevant polymorphisms in thyroid hormone pathway genes. In this study, we focused on the D1, D2, D3, TSHR, and TRβ genes and identified eight polymorphisms of interest. Four of them (D2-Thr92Ala, TSHRa-
Asp36His, TSHRb-Pro52Thr, and TSHRc-Asp727Glu) have been reported previously (21, 25–27). This is the first study that reports the polymorphisms D1a-C/T, D1b-A/G, D3-T/G, and TR/H9252-T/C. Three are located in the 3'/H11032-UTR of the deiodinase genes, i.e., D1a-C/T, D1b-A/G, and D3-T/G, whereas one, TR/H9252, is a silent SNP. No other polymorphisms have been reported in any of the three deiodinases so far.

We found a significant correlation between the D1a-C/T polymorphism and plasma rT3, with rT3 levels progressively increasing in subjects with zero, one, or two D1a-T alleles. The T variant was also positively correlated with the plasma rT3/T4 ratio, and negatively correlated with the T3/rT3 ratio. Although the D1b-A/G polymorphism was not significantly correlated with plasma rT3, the G variant was negatively correlated with the rT3/T4 ratio and positively with the T3/rT3 ratio. Liver D1 plays a key role in the production of plasma T3 from T4 and in the breakdown of the metabolite rT3 (7, 8). rT3 is by far the preferred substrate for D1 (7, 8). For this reason, a functionally relevant SNP in D1 is expected to affect plasma iodothyronine levels, in particular rT3, and ratios between plasma iodothyronines (7, 8). These data suggest a negative effect of the D1a-T variant on total D1 activity, whereas the D1b-G variant appears to have a positive effect. This is supported by the haplotype analysis, which showed a negative relation of the C-G haplotype allele (haplotype 3) and a positive relation of the T-A haplotype allele (haplotype 2) with plasma rT3 and rT3/T4 levels. These haplotype alleles had an opposite effect on plasma T3/rT3 levels. Because both SNPs are located in the 3'-UTR, a change in the stability of the mRNA is an attractive explanation. Another explanation may be an altered folding of the mRNA, which—at least at the site of the SECIS element—is necessary for the incorporation of the SeC. Alternatively, the association could be explained by linkage disequilibrium with another SNP located in the coding sequence or in regulatory areas of the gene, such as

<table>
<thead>
<tr>
<th>Deiodinase</th>
<th>Km (T4) (nM)</th>
<th>Vmax (T4) (pmol T4/min/mg)</th>
<th>Vmax/Km</th>
<th>Km (rT3) (nM)</th>
<th>Vmax (rT3) (pmol rT3/min/mg)</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2-WT</td>
<td>4.3/3.1</td>
<td>0.32/0.61</td>
<td>0.07/0.2</td>
<td>17</td>
<td>0.34</td>
<td>0.02</td>
</tr>
<tr>
<td>D2-Thr92Ala</td>
<td>5.5/3.3</td>
<td>0.49/0.89</td>
<td>0.09/0.22</td>
<td>19</td>
<td>0.38</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Results of two separate experiments are shown, using T4 as a substrate.
the promoter region of the D1 gene. However, in this study we did not find any evidence for SNPs in the coding sequence of D1.

We did not see a significant correlation of the D1a and D1b SNPs with plasma T3 concentration, which can be explained by the fact that there are many pathways for T3 metabolism, such as glucuronidation, sulfation, and deiodination by D1 and D3. In addition, there are three sources of plasma T3, i.e. thyroidal T4 secretion, outer ring deiodination (ORD) of T4 by D1, and ORD of T3 by D2 (7, 28). On the other hand, rT3 turnover is less complicated; it is largely produced by inner ring deiodination of T4 by D3, and cleared by D1-catalyzed ORD. This may explain why no correlation was found of the SNPs in D1 with plasma T3, whereas we did find a significant correlation with rT3 levels.

Local D2 activity is essential for the negative feedback regulation of hypophyseal TSH secretion by plasma T4, as demonstrated in the D2 knockout mouse (19). No significant effect of the D2-Thr92Ala polymorphism was seen on plasma TSH levels, iodothyronine levels or their ratios in this healthy population. However, D2 is particularly important for local T3 production in D2-containing tissues (7, 8), and on the basis of this study, it cannot be excluded that intracellular thyroid hormone levels are affected by this polymorphism.

The D2-Ala92 variant was previously reported to be associated with insulin resistance (25). However, the functional consequences of this polymorphism with regard to deiodinase activity were not investigated in this study (25). To determine the possible functional effects of this polymorphism, we performed in vitro analysis of the D2-Thr92Ala variant by transfection of COS cells. In an attempt to mimic the heterozygous situation, cells were also transfected with a combination of both variants. No significant differences in D2 activity were observed, either when assays were done on homogenates in the presence of excess DTT as cofactor or in intact cells in the presence of the natural cofactor of the enzyme. Whether the previously reported association (25) can be explained by linkage with another polymorphism and whether this association can be confirmed in different populations should be explored in future studies.

D3 is present in human brain, placenta, skin, and various fetal tissues, and is the major T3 and T3-inactivating enzyme by catalyzing their conversion to rT3 and 3,3′-T2, respectively. It plays an essential role during fetal development in which it protects the embryo from excess thyroid hormone (29). We did not find any effect of the D3-T/G polymorphism on plasma iodothyronine levels in this population. The effects of this polymorphism on tissue thyroid hormone levels, and, thus, for instance on brain development and function, should be explored in future studies.

The growth and function of the thyroid are controlled by TSH through its receptor (30). Many mutations in TSHR have been identified as causes of thyroid diseases (30, 31). In addition to these mutations, three interesting polymorphisms in the coding sequence of the TSHR gene have been described. TSHRa-Asp36His and TSHRb-Pro52Thr polymorphisms are situated in the extracellular domain of the receptor, whereas the TSHRc-Asp727Glu polymorphism is located in the intracellular domain. The His variant of the TSHRa-Asp36His polymorphism was detected in only two subjects in our population, indicating an allelic frequency of less than 1%, which is not significantly different from the frequency reported by Gustavsson et al. (26). No further statistical analysis was performed for this polymorphism.

The TSHRb-Pro52Thr polymorphism was more frequent, with an allelic frequency of the Thr variant of 6%, which is in agreement with other studies (21, 32). The genotype distribution of this SNP is not in Hardy-Weinberg equilibrium, which may be explained by the small size of the groups or by multiple testing, rather than by population bias. Conflicting observations regarding the response of this variant to TSH stimulation (33–35) could reflect the subtle effects of this polymorphism. We found no association of this polymorphism with plasma TSH levels, which can be due to the low frequency of the SNP. This SNP should therefore be analyzed in future studies in larger populations.

The allele frequency of the Glu variant of the TSHRc-Asp727Glu polymorphism is 9.5%, in agreement with results reported by Nogueira et al. (36). Conflicting data have been obtained regarding the cAMP response to TSH of this variant receptor in transfection studies (36, 37). No evidence was found for an association of the TSHRc-Asp727Glu polymorphism with nonautoimmune hyperfunctioning thyroid disorders (27). However, we found a significant association of the TSHRc-727Glu variant with lower plasma TSH levels and, although not significant, with lower plasma TSH/T4 and TSH/FT4 ratios. These data agree with findings showing an increased cAMP response of the TSHRc-Glu variant (37) to TSH, because less TSH would be required to achieve a normal thyroid hormone production. Together, these data suggest that this is a functionally relevant SNP.

We identified a silent polymorphism in the coding sequence of the TRβ gene (T1536C), with an allelic frequency of the C variant of 3%. No evidence was found in our population for an association of the TRβ-C/T polymorphism with plasma TSH or iodothyronine levels, or their ratios. Due to the low number of subjects with the TRβ-T variant, no conclusions can be drawn from our observations regarding the (lack of) functional importance of this polymorphism.

In conclusion, we have analyzed the possible association of eight SNPs in five thyroid hormone pathway genes with plasma TSH and iodothyronine levels. Four of those polymorphisms have not been described before. We find significant effects for three SNPs on plasma TSH and/or thyroid hormone levels. Due to the low frequency of several other SNPs, no conclusions can be drawn in our current population about their effect on plasma TSH and/or thyroid hormone levels.

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