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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 T_3 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 T_4, free T_4, T_3, rT_3, 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-T/G (T = 85.5%, G = 14.2%). Four are missense SNPs: D2-A/G (Thr92Ala, Thr = 61.2%, Ala = 38.8%), TSHRa-G/C (Asp36His, Asp = 99.4%, His = 0.6%), TSHRb-C/A (Pro52Thr, Pro = 94.2%, Thr = 5.8%), and TSHRc-C/G (Asp727Glu, Asp = 90.7%, Glu = 9.3%). One is a silent SNP: TRβ-T/C (T = 96.8%, C = 3.2%). D1a-T was associated in a dose-dependent manner with a higher plasma rT_3 [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 rT_3/T_4 ($P = 0.01$), and a lower T_3/rT_3 ($P = 0.003$) ratio. The D1b-G allele was associated with lower plasma rT_3/T_4 ($P = 0.024$) and with higher T_3/rT_3 ($P = 0.08$) 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 T_4 ($P = 0.06$), TSH/T_3 ($P = 0.06$), and TSH/T_4 ($P = 0.08$) 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 T_3 via a mechanism of T_3-regulated gene expression (1). Examples are the effects of thyroid hormone on brain development and skeletal maturation, on heat production and oxygen consumption, 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 T_4, is controlled by the classic hypothalamic-pituitary-thyroid axis, whereas the biological activity of thyroid hormone, *i.e.* the availability of T_3, 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 T_3 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

Abbreviations: D1–D3, Deiodinases; dbEST, EST database; dbSNP, SNP database; DTT, dithiothreitol; EST, expressed sequence tag; FT_4, free T_4; ORD, outer ring deiodination; RFLP, restriction fragment length polymorphism; SBE, single base extension; SeC, selenocysteine; SECIS, SeC insertion sequence; SNP, single nucleotide polymorphism; TRβ, T_3 receptor β; TSHR, TSH receptor; UTR, untranslated region; WT, wild-type.

the genotype was correlated with a specific set of plasma thyroid indexes.

For the SNPs in the three deiodinases, we analyzed correlations with plasma T_4, T_3, and rT_3 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 hypophyseal TSH secretion by plasma T_4, as demonstrated in the D2 knockout mouse (19). Therefore, the relation between the D2 SNP and plasma TSH, and TSH/T_4 and TSH/free T_4 (FT_4) 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 FT_4 and TSH levels indicating hyperthyroidism, another because of hypothyroid FT_4 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 ± 12.2 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 blood 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

Parameter	Mean ± SD[a]	Reference values[b]
n	155	
Age	46.3 ± 12.2[c]	
TSH (mU/liter)	1.32 ± 0.75[d]	0.4–4.3
T_4 (nmol/liter)	87.96 ± 15.9	58–128
FT_4 (pmol/liter)	15.06 ± 2.4	11–25
T_3 (nmol/liter)	1.96 ± 0.24	1.43–2.51
rT_3 (nmol/liter)	0.31 ± 0.08	0.14–0.34

[a] TSH and iodotyronine levels of this population were determined in plasma.
[b] Reference values used in our lab, with TSH and iodothyronine levels determined in serum.
[c] n = 156, [d] n = 154.

Plasma analyses

Plasma T_4, FT_4, T_3, and TSH were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, UK). rT_3 was measured by RIA as previously described (20).

DNA isolation

DNA was extracted from 2 ml of blood using the PUREGENE DNA Isolation Kit (Gentra 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/liter (stock) and 10 ng/μl (work solution). Purity was determined by measuring the 260/280-nm ratio.

Sequence analysis

DNA from 15 random subjects was used for sequence analysis. The SECIS-element and flanking regions were analyzed in the D1, D2, and D3 genes. At first, DNA was amplified by PCR using the primers listed in Table 2. All primers used in this study were ordered from Invitrogen (Breda, The Netherlands). The PCR was performed in a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), and conditions were 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 High Pure PCR Product 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., Veenendaal, 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.nlm.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 D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRa-Asp/His, TSHRb-Pro/Thr, and TSHRc-Asp/Glu 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 *Rsa*I. For D3-T/G and TSHRb-C/A, mismatch primers were used to generate restriction sites for *Bss*SI and *Tth* 111 I, 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 contained 1× PCR buffer (Invitrogen), 0.2 mM of each

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

Primers		PCR	Annealing temperature	Sequencing PCR product
SECIS-D1	Fw	5′-TGAAATCTTCCACTAGCCTCA-3′	50C	5′-CAACAGAGTCATCTAGAAGGGA-3′
	Rv	5′-ACTCCAGCAGGAGTTCATAAG-3′		5′-CACATTTAACATGTAACATAG-3′
SECIS-D2	Fw	5′-AAGCCAACAGGTAAACACATA-3′	45C	5′-CCAGGCCCATGTCACTGGTCA-3′
	Rv	5′-AATACAAGAACAAATTGTATA-3′		5′-CACACATAGCACTCAGCACCA-3′
SECIS-D3	Fw	5′-AGTGCTTTGCCCGGTGCTTC-3′	50C	5′-GCTGCACAGAGACCTTGGCCA-3′
	Rv	5′-CACCAAATGGCCTTCGAATCA-3′		5′-AATCAGCAGCTCTGCCTAGGA-3′
D3-CDS1	Fw	5′-CCCAGATGCCTCGCCAGGCCA-3′	58C	5′-TGGTCGGAGAGGGCGAGGGGTC-3′
	Rv	5′-GCGCTCATGCGCGCCATGAA-3′		5′-TGCAGCTGCCGAAATTGAGAA-3′
D3-CDS2	Fw	5′-TTCTTCAAGCAGGCGCACGA-3′	52C	5′-CTCCGAGGTGGTTCTGCCCGA-3′
	Rv	5′-CGCTTGCACGTGGGCTTCGAA-3′		5′-CTCGAAGGCCCAGCCCACCAA-3′

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

		RFLP primers	Annealing temperature	Restriction enzymes		SBE primers
D1a-C/T	Fw	5′-GAACTTGATGTGAAGGCTGGA-3′	54C	*Bcl*I	Fw	5′-(T_4)GATACCTGAATTCTAGGTGA-3′
	Rv	5′-TAACCTCAGCTGGGAGTTGTTT-3′			Rv	5′-(T_4)CTTGAGAAGCCCTCCCGTTG-3′
D1b-A/G	Fw	5′-CAACAGAGTCATCTAGAAGGGA-3′	48C	*Spe*I	Fw	5′-(T_{10})TTATAAGATGCAGTAAACTA-3′
	Rv	5′-CACATTTAACATGTAACATAG-3′			Rv	5′-(T_{10})ATATTTTCATCATTCTGTTA-3′
D2-A/G	Fw	5′-GATAGTAAAGAATAACAGCCTTGGCT-3′	58C	*Rsa*I	Fw	5′-(T_{16})GTGTGGTGCATGTCTCCAGT-3′
	Rv	5′-CAGCTATCTTCTCCTGGATACCA-3′			Rv	5′-(T_{16})ACTGTTGTCACCTCCTTCTG-3′
D3-T/G	Fw	5′-CTGGTAGGGGAAGTGATCTCG-3′	60C	*Bss*SI	Fw	5′-(T_{22})TGGTAGGGGAAGTGATGTCG-3′
	Rv	5′-GCCAATGCCTCTCAAGCTATC-3′			Rv	5′-(T_{22})CTGCCCACCCCTCCCCATCC-3′
TSHa-G/C	Fw	5′-ATTTCGGAGGATGGAGAAATA-3′	53C	*Bsp* 1286I		
	Rv	5′-GTCTGCGTACTGGGCGGTAA-3′				
TSHb-C/A	Fw	5′-GCGATTTCGGAGGATGGAGAAATAGC-3′	58C	*Tth*111I	Fw	5′-(T_{28})AACGCATCCCCAGCTTACCG-3′
	Rv	5′-CCGGGTACTCACAGAGTCTGCGACCTG-3′			Rv	5′-(T_{28})CTTCAGAGTCTGCGTACTGG-3′
TSHc-C/G	Fw	5′-AACGCCAGGCTCAGGCATAC-3′	60C	*Nla*III	Fw	5′-(T_{34})GTTCAAAAGGTTACCCACGA-3′
	Rv	5′-AAGTTCCCCTACCATTGTGA-3′			Rv	5′-(T_{34})TTGTGGAGACCCTGCCTCAT-3′
TRβ-T/C					Fw	5′-(T_{40})AAACACCACGTGACACACTT-3′
					Rv	5′-(T_{40})TTCATCAGGAGTTTAGGCCA-3′

Mismatches are shown by an *underscore*.

deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 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-T/G, TSHRa-Asp/His, TSHRb-Pro/Thr, and TSHRc-Asp/Glu polymorphisms using RFLP (see Fig. 2 for examples).

Single base extension (SBE) analysis

To validate the genotypes, two independent methods were used. In addition to RFLP, genotyping was also performed by SBE analysis. All subjects were genotyped for the D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRb-Pro/Thr, TSHRc-Asp/Glu, and TRβ-T/C polymorphisms using SBE. PCR products were generated using the same primers as used for RFLP analysis (Table 3), except for TSHRb and TRβ. A PCR fragment of TSHR1b was generated using 5′-ATTTCGGAGGATGGAGA-AATA-3′ as the forward primer and 5′-GCAGATGCCCTTGAT-CTCTG-3′ as the reverse primer. Annealing temperature was 58 C. For TRβ1, 5′-TGCCTGTGTTGAGAGAATAG-3′ was used as the forward primer and 5′-GTCTAATCCTCGAACACTTC-3′ as the reverse primer; annealing temperature was 51 C. All other conditions were the same as mentioned above for the other PCRs. 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. For SBE analysis of the D1a, D3, and TSHRb SNPs, the reverse primers were used, and for the other SNPs the forward primers were used.

Functional characterization of D2-Thr92Ala variant

A human D2 expression vector (pcDNA3-hD2-rSECIS), containing the human D2 cDNA inserted 5′ to the SECIS element of the rat D1 gene, was used as template for site-directed mutagenesis via the circular mutagenesis procedure (22, 23). Overlapping sense and antisense primers containing the nucleotide changes needed to produce the Thr92Ala D2 mutant (sense 5′-GTGCATGTCTCCAGTGCAGAAGGAGGT-GACAAC) cDNA were used in circular mutagenesis reactions with 50 ng plasmid template and 2 U Pfu DNA polymerase (23). Plasmid DNA isolated from five clones was sequenced directly on an automated ABI 310 capillary sequencer to verify that the desired mutation had been generated and that no spurious mutations had occurred during amplification.

The WT and variant D2 enzymes were expressed in COS cells (65-cm^2 dishes) by diethylaminoethane-dextran-mediated transfection with 8 μg plasmid DNA (22). After 2 d, the cells were rinsed with PBS and collected in 0.25 ml PE buffer [0.1 M phosphate (pH 7.2), 2 mM EDTA], sonicated, aliquoted, and stored at −80 C.

The principle of the *in vitro* D2 activity assay is the production of radioiodide by outer-ring deiodination of [3′,5′-^{125}I]T_4 or [3′,5′-^{125}I]rT_3. Duplicate incubations contained about 100,000 cpm labeled T_4 or rT_3 with varying amounts of unlabeled substrate (T_4 or rT_3) and COS cell homogenates (50–100 μg protein) in a total volume of 0.2 ml of PED20 buffer [20 mM dithiothreitol (DTT)]. Mixtures were incubated for 60 min at 37 C, whereafter the production of radioiodide was determined as described (22).

The principle of the *in situ* D2 activity assay is the release of [^{125}I]T_3 in the medium of D2 expressing COS-1 cells incubated with [3′,5′-^{125}I]T_4. 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 [^{125}I]T_4 (10^6 cpm/ml) and 1–1000 nM unlabeled T_4. 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 ± SD in the tables. Comparison of the frequencies of the genotypes between males and females was carried out using a χ^2 test. Deviation from Hardy-Weinberg equilibrium was also analyzed using a χ^2 test. *P* values are two-sided throughout, and *P* less than 0.05 was considered significant. Haplotype 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 SECIS 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 SECIS element. This variation was confirmed in the dbEST. No

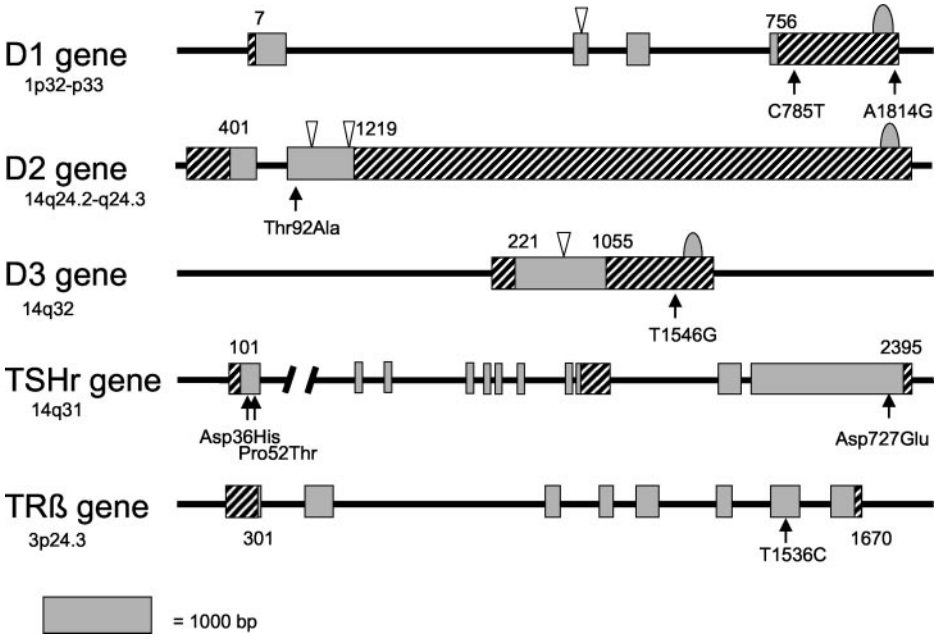

FIG. 1. The thyroid hormone pathway genes analyzed in this study and in which polymorphisms were identified. Nucleotide numbers are based on the following GenBank accession no.: 4557521 for D1, 13654872 for D2, 4503334 for D3, 4507700 for TSHR, and 10835122 for TRβ. Size of the different exons is indicated by their scale. The coding sequence is represented by ▨, whereas ▨ indicates the UTR. A UGA codon, coding for SeC, is depicted by [▽]. Finally, SECIS elements are indicated by ⌓.

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 gene (TRβ-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).

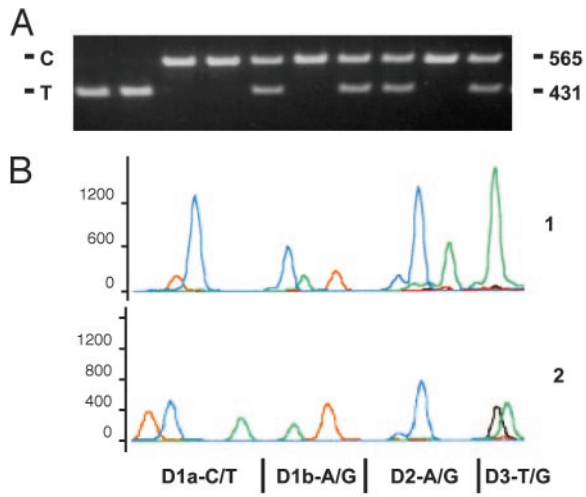

FIG. 2. A, RFLP analysis of 10 subjects for D1a-C/T. A PCR fragment was generated of 565 bp. Incubation with *Bcl*I generates two fragments of 434 and 131 bp only in the presence of the D1a-T allele (only the 565- and 434-bp fragments are shown in this figure). B, SBE analysis of two subjects for D1a-C/T, D1b-A/G, D2-A/G, D3-T/G. *Orange peaks* represent the size marker, *blue peaks* represent a C, *green peaks* represent a T, and a *black peak* represents a G. The reverse primer was used for D1b-A/G and D2-A/G (see *Materials and Methods*). By developing primers of a different size, it is possible to analyze several SNPs in one SBE reaction. Subject 1 is heterozygous for D1b-A/G and D2-A/G, whereas subject 2 is heterozygous for D1a-C/T and D3-T/G.

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 rT_3 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

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

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

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

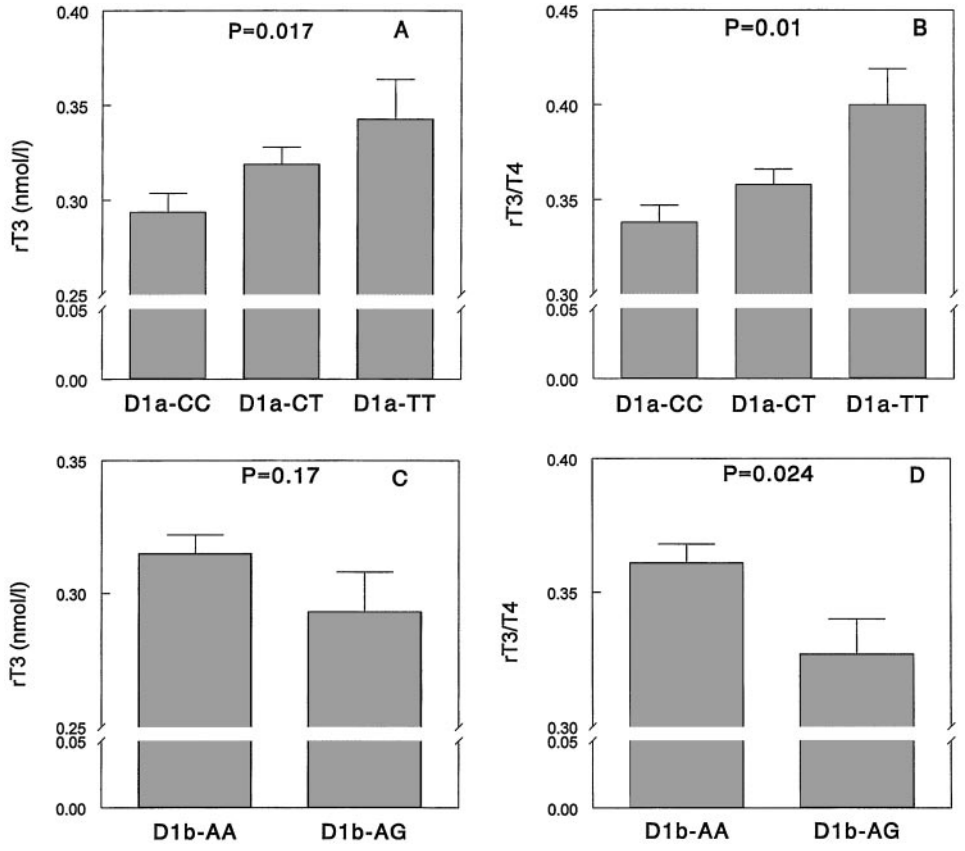

FIG. 3. Differences between plasma rT_3 concentrations (A and C) and plasma rT_3/T_4 ratios (B and D) by genotype for the D1a (A and B) and D1b (C and D) polymorphism. *P* values represent an ANOVA test (C) or an ANOVA test for linearity (A and B). To convert values for rT_3 to nanograms per deciliter, multiply by 65.1.

the T-allele to be associated with increasing plasma rT_3/T_4 ratio ($P = 0.01$; Fig. 3B) and decreasing plasma T_3/rT_3 ratio ($P = 0.003$). The G allele of the D1b-A/G polymorphism was less frequent and was associated with lower plasma rT_3/T_4 ($P = 0.024$) and higher T_3/rT_3 ($P = 0.08$) ratios. Due to the low frequency of the polymorphism, the difference in rT_3 levels failed to reach significance ($P = 0.17$; Fig. 3, C and D).

These two D1 SNPs were analyzed for linkage disequi-

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 rT_3, rT_3/T_4, or with T_3/rT_3 ratio. The haplotype 2 showed a positive correlation with rT_3 ($P = 0.017$) and rT_3/T_4 ratio ($P = 0.01$), and a negative relation with T_3/rT_3 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 rT_3 ($P = 0.172$) and with rT_3/T_4 ($P = 0.024$) and a positive relation with T_3/rT_3 ($P = 0.08$) 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/T_4 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/FT_4 ($P = 0.06$), TSH/T_4 ($P = 0.08$), and TSH/T_3 ($P = 0.06$) ratios than subjects homozygous for the Asp allele.

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

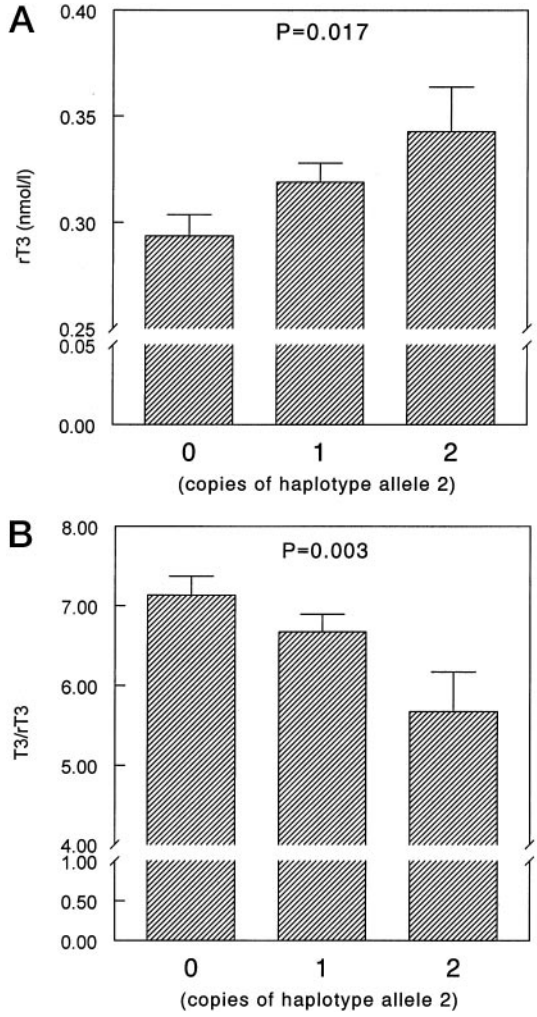

FIG. 4. Differences in rT_3 levels (A) and T_3/rT_3 ratios (B) in subjects analyzed by genotype for haplotype alleles for the combined D1a and D1b polymorphisms (1 = C–A, 2 = T–A, 3 = C–G). *P* values represent an ANOVA test for linearity. To convert values for rT_3 to nanograms per deciliter, multiply by 65.1.

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 K_m and V_{max} values using T_4 or rT_3 as substrate (Table 5 and Fig. 6A). Upon incubation of intact transfected cells with increasing amounts of unlabeled T_4, the fractional deiodination of $[^{125}I]T_4$ decreased in a similar way (Fig. 6B). No differences in deiodination of T_4 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-

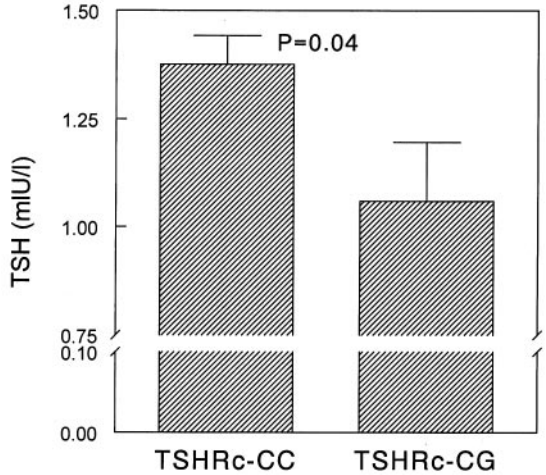

FIG. 5. Difference in plasma TSH concentration between subjects homozygous for the TSHRc-Asp (TSHRc-C) variant and subjects heterozygous for the TSHRc-Asp/Glu (TSHRc-C/G) variants ($P < 0.05$).

TABLE 5. Kinetic characteristics of D2 enzymes (incubations in presence of 20 mM DTT)

Deiodinase	K_m (T_4) (nM)	V_{max} (T_4) (pmol T_4/min·mg)	V_{max}/K_m	K_m (rT_3) (nM)	V_{max} (rT_3) (pmol rT3/min·mg)	V_{max}/K_m
D2-WT	4.3/3.1[a]	0.32/0.61[a]	0.07/0.20[a]	17	0.34	0.02
D2-Thr92Ala	5.5/3.1[a]	0.49/0.69[a]	0.09/0.22[a]	19	0.38	0.02

[a] Results of two separate experiments are shown, using T_4 as a substrate.

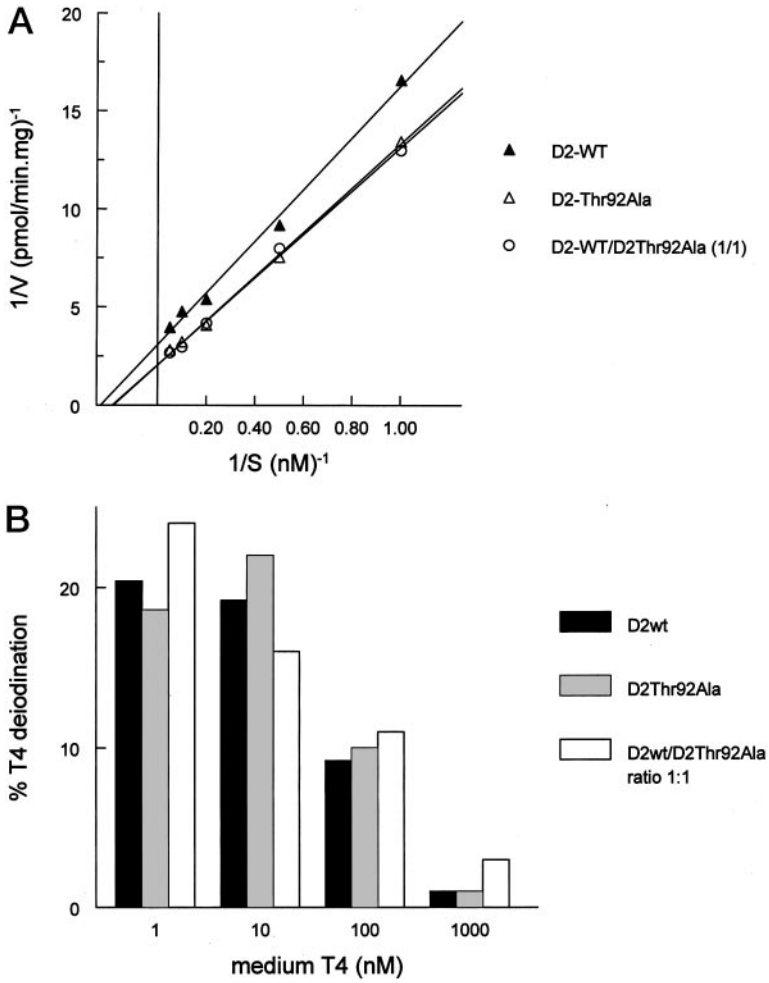

FIG. 6. A, Lineweaver-Burk plot of T_4 deiodination by D2-WT, D2-Thr92Ala, or D2-WT/D2Thr92Ala (1:1) at 20 mM DTT in the presence of varying concentrations of T_4. B, *In situ* deiodination at varying T_4 concentrations. COS cells were transfected with D2-WT, D2-Thr92Ala, or D2-WT/D2Thr92Ala (1:1) expression vectors, and intact cell deiodination was determined as described in *Subjects and Methods*.

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 TRβ-T/C. Three are located in the 3′-UTR of the deiodinase genes, *i.e.* D1a-C/T, D1b-A/G, and D3-T/G, whereas one, TRβ-T/C, 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 rT_3, with rT_3 levels progressively increasing in subjects with zero, one, or two D1a-T alleles. The T variant was also positively correlated with the plasma rT_3/T_4 ratio, and negatively correlated with the T_3/rT_3 ratio. Although the D1b-A/G polymorphism was not significantly correlated with plasma rT_3, the G variant was negatively correlated with the rT_3/T_4 ratio and positively with the T_3/rT_3 ratio. Liver D1 plays a key role in the production of plasma T_3 from T_4 and in the breakdown of the metabolite rT_3 (7, 8). RT_3 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 rT_3, 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 rT_3 and rT_3/T_4 levels. These haplotype alleles had an opposite effect on plasma T_3/rT_3 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

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 T_3 concentration, which can be explained by the fact that there are many pathways for T_3 metabolism, such as glucuronidation, sulfation, and deiodination by D1 and D3. In addition, there are three sources of plasma T_3, *i.e.* thyroidal T_3 secretion, outer ring deiodination (ORD) of T_4 by D1, and ORD of T_4 by D2 (7, 28). On the other hand, rT_3 turnover is less complicated; it is largely produced by inner ring deiodination of T_4 by D3, and cleared by D1-catalyzed ORD. This may explain why no correlation was found of the SNPs in D1 with plasma T_3, whereas we did find a significant correlation with rT_3 levels.

Local D2 activity is essential for the negative feedback regulation of hypophyseal TSH secretion by plasma T_4, 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 T_3 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 T_4 and T_3-inactivating enzyme by catalyzing their conversion to rT_3 and 3.3′-T_2, 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/T_4, and TSH/FT_4 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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