A large-scale association analysis of 68 thyroid hormone pathway genes with serum TSH and FT4 levels

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Abstract

Objective: Minor variation in serum thyroid hormone (TH) levels can have important effects on various clinical endpoints. Although 45–65% of the inter-individual variation in serum TH levels is due to genetic factors, the causative genes are not well established. We therefore studied the effects of genetic variation in 68 TH pathway genes on serum TSH and free thyroxine (FT4) levels.

Design and methods: Sixty-eight genes (1512 polymorphisms) were studied in relation to serum TSH and FT4 levels in 1121 Caucasian subjects. Promising hits (P<0.01) were studied in three independent Caucasian populations (2656 subjects) for confirmation. A meta-analysis of all four studies was performed.

Results: For TSH, eight PDE8B polymorphisms (P=4.10^-17) remained significant in the meta-analysis. For FT4, two DIO1 (P=8.10^-12) and one FOXE1 (P=0.0003) polymorphisms remained significant in the meta-analysis. Suggestive associations were detected for one FOXE1 (P=0.0028) and three THRB (P=0.045) polymorphisms with TSH, and one SLC16A10 polymorphism (P=0.0110) with FT4, but failed to reach the significant multiple-testing corrected P value (P<0.0022 and P<0.0033 respectively).

Conclusions: Using a large-scale association analysis, we replicated previously reported associations with genetic variation in PDE8B, THRB, and DIO1. We demonstrate effects of genetic variation in FOXE1 on serum FT4 levels, and borderline significant effects on serum TSH levels. A suggestive association of genetic variation in SLC16A10 with serum FT4 levels was found. These data provide insight into the molecular basis of inter-individual variation in TH serum levels.

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Introduction

Adequate thyroid hormone (TH) levels are essential for normal growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all human tissues. Epidemiological evidence shows that minor variation in TH serum levels, even within the normal range, can have important effects on clinical endpoints, such as bone mineral density (1), atrial fibrillation (2), metabolic syndrome (3), and cardiovascular mortality (4).

In healthy subjects, serum TSH and TH levels show substantial inter-individual variability, leading to wide laboratory reference ranges, whereas the intra-individual variability is within a narrow range, suggesting that every person has his/her own individual ‘set-point’ (5). Approximately 45–65% of this inter-individual variation in serum TSH and TH levels is due to genetic factors (6, 7). The causative genes are, however, not well established. Well-known TH pathway genes such as the deiodinases (8–12), TSH receptor (10, 13, 14) and TH transporters (15–17) have been associated with TH serum levels, but their contribution to the overall variation is modest (12, 13). A genome-wide linkage scan by Panicker et al. (18) identified eight chromosomal loci involved in the control of the pituitary–thyroid axis, but as can be expected from this type of study, the actual genes were not identified. Recent genome-wide association studies demonstrated associations of polymorphisms located in phosphodiesterase 8B (PDE8B; 19) and the CAPZB locus (20) with serum TSH levels.
The identification of new associations in genome-wide association studies is hampered by the need for stringent correction for multiple testing, requiring \( P \) values < \( 5 \times 10^{-7} \) (21). For this reason, we performed a focused association analysis of 68 candidate genes, known to be involved in TH synthesis, metabolism or transport, in relation to serum TSH and free thyroxine (FT\(_4\)) levels. Promising hits were studied in three independent populations for confirmation.

Materials and methods

The association of serum TSH and FT\(_4\) levels with genetic variation in the candidate genes was studied in the Rotterdam Study (22). Promising associations were studied in a Danish twin population (6), the Scan Study (23), and the Nijmegen Biomedical Study (24) for confirmation, after which a meta-analysis of all four studies was conducted.

Subjects with serum FT\(_4\) levels indicating hypo- or hyper-thyroidism were excluded, as common genetic variation is in general thought to play a minor role in the pathogenesis of hypo- and hyper-thyroidism. As the role of common genetic variation in subclinical hypo- or hyper-thyroidism is less clear, we did not exclude subjects with TSH levels outside the reference ranges. Positive thyroid peroxidase antibodies (TPOAbs), known thyroid disease, and/or thyroid medication usage were excluded from all analyses.

Selection of candidate genes

A selection of candidate genes was made by searching NCBI GenBank for ‘thyro’ limited by ‘human’ and ‘current’ (520 genes). Based on the current literature, an expert in the field (T J V) reviewed these genes, which resulted in a selection of 70 genes with a known role in TH synthesis, transport or metabolism. As our cohorts consisted of both men and women, genes on the X-chromosome (i.e. monocarboxylate transporter 8 (MCT8) and TBG) were excluded, resulting in a final selection of 68 genes (Fig. 1).

Study populations

The Rotterdam Study is a prospective population-based cohort study on determinants of chronic diseases in the elderly (22). The study comprised of 7983 men and women living in a district of Rotterdam, The Netherlands. Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. At baseline, all participants were interviewed and underwent extensive physical examination. Serum TSH (TSH Lumitest; Henning, Berlin, Germany), FT\(_4\) (chemoluminescence assay; Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK), and TPOAb (ELISA; Milenia, DPC, Los Angeles, CA, USA) levels were determined in 1350 subjects of whom DNA was available. After excluding subjects with serum FT\(_4\) levels indicating hypo- or hyper-thyroidism, positive TPOAbs, known thyroid disease, and/or thyroid medication usage, 1121 subjects were available for analysis.

The Danish twin population is part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on various traits related to the metabolic syndrome and cardiovascular risk factors. Rationale and design have been described in detail previously (6). In short, a representative sample of self-reported healthy twin pairs was recruited from the population-based Danish Twin...
Registry (25). In the GEMINAKAR study, 1512 men and women (756 twin pairs) were examined. Informed consent was obtained from each participant, and all regional Danish Scientific-ethical committees approved the study. At baseline, all participants were interviewed and underwent physical examination. Serum TSH (fluoroenzymometric assay; PerkinElmer/Wallac, Turku, Finland), FT₄ (AutoDELFIA; PerkinElmer/Wallac), and TPOAb (AutoDELFIA; PerkinElmer/Wallac) levels were determined in 905 subjects of whom DNA was available. After applying exclusion criteria, 474 unrelated subjects were included in the present study.

The Scan Study is a prospective population-based cohort study in 1077 men and women, designed to study causes and consequences of age-related brain changes on MRI. Rationale and design have been described in detail previously (23). Informed consent was obtained from each participant, and the Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. At baseline, all participants were interviewed and underwent physical examination. Serum TSH, FT₄ (chemoluminescence assay; Vitros, ECI Immunodiagnostics System, Ortho-Clinical Diagnostics, Rochester, NY, USA), and TPOAb (immunometric assay; DPC) levels were determined in 854 subjects of whom DNA was available (8). After applying exclusion criteria, 697 subjects were included in the present study.

The Nijmegen Biomedical Study is a population-based survey on lifestyle and medical history in 9350 men and women living in Nijmegen, The Netherlands. Rationale and design have been described previously (24). Informed consent was obtained from each participant, and the Institutional Review Board of the Radboud University Nijmegen Medical Centre approved the study. Data on serum TSH (immunoluminometric assay; Architect, Abbott Diagnostics Division), FT₄ (chemoluminescence assay; Vitros, ECI Immunodiagnostics System, Ortho-Clinical Diagnostics) and TPOAb levels (fluoroenzymometric assay; Abbott Diagnostics Division), and Illumina HumanHap370K array (see below) genotype data were available from 1832 subjects (24). After applying exclusion criteria, 1485 subjects were included in the present study.

Genotyping

In all study populations, genomic DNA was extracted from samples of peripheral venous blood, according to standard procedures. Subjects in the Rotterdam Study were genotyped using the Illumina HumanHap550K array. All directly genotyped polymorphisms with a minor allele frequency (MAF) ≥ 5% and located within a 20 kb region (10 kb upstream to 10 kb downstream) of each of the 68 candidate genes were selected. After quality control (QC) and exclusion of polymorphisms with a Hardy–Weinberg equilibrium (HWE) P value ≤ 1 × 10⁻⁶ or genotyping call rate < 90%, 1512 polymorphisms were included in the analysis.

Genotypes for replication (46 polymorphisms) were determined using PCR, iPLEX single base primer extension, and matrix-assisted laser desorption/ionization – time of flight mass spectrometry in a 384-well format (Sequenom, San Diego, CA, USA; see: http://www.sequenom.com) and ABI Taqman allelic discrimination Assay-on-Demand (Applied Biosystems, Inc., Foster City, CA, USA). Polymorphisms with a genotyping call rate < 90% or a deviation from HWE were excluded from the analyses. rs989758, rs13097208, rs13066296, and rs832790 had a genotyping call rate < 90% in both the Danish twin population and the Scan Study. For the Nijmegen Biomedical Study, genotype data were available from the Illumina HumanHap370K array (26). Polymorphism imputation after QC was based on Phase II CEU HapMap samples (version 22, build 36) and was done using IMPUTE (27).

Statistical methods

In the Rotterdam Study, associations with serum TSH and FT₄ levels were assessed by linear regression using gender- and age-adjusted standardized residuals in PLINKv1.07 (28) and SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). Due to non-normal distribution, TSH was transformed by the natural logarithm. Polymorphisms that showed significant associations at P < 0.01 were genotyped and studied in the Danish twin population, the Scan Study, and the Nijmegen Biomedical Study for confirmation, using SPSS 15.0 for Windows and SNPTEST (27). To minimize the influence of inter-assay variation, effect sizes were assessed by linear regression using gender- and age-adjusted standardized residuals. For the polymorphisms that did not reach the P value threshold of P < 0.01 in the Rotterdam Study, there was 80% power to detect differences of 0.23, 0.17, and 0.15 S.D. in TSH and FT₄ levels for MAFs of 10, 20, and 30% respectively. This study is therefore powered to detect at least moderate effects.

Meta-analyses based on all four populations were conducted using the METAL software package applying inverse-variance weighted fixed-effects methodology (http://www.sph.umich.edu/csg/abecasis/Metal). To control for multiple testing, a P value threshold for both the TSH and the FT₄ meta-analyses was calculated based on the number of independent polymorphisms tested, thereby taking the linkage disequilibrium (LD) structure between these polymorphisms into account. The number of independent polymorphisms was calculated in the Rotterdam Study, using PLINKv1.07 (28), for which a LD threshold of r² > 0.2 was used. To define a P value threshold to declare statistical significance, we divided P = 0.05 by the number of independent tests, which was estimated to be 22.84 for...
TSH and 15.24 for FT4. Consequently, the $P$ value thresholds for the TSH and FT4 meta-analyses were respectively $P=0.0022$ and $P=0.0033$.

**Results**

Baseline characteristics of the studied populations are shown in Table 1.

**Figure 1** summarizes the flowchart for the TSH and FT4 analyses, together with the genes that passed selection at each stage of the study. Of the 1512 studied polymorphisms in 68 genes, 30 polymorphisms in 10 genes showed a significant association (i.e. $P<0.01$) with TSH in the Rotterdam Study. Of these, eight polymorphisms in **PDE8B** remained significant at $P<0.0022$ in the meta-analysis of the four populations (Table 2). Suggestive associations were detected for one polymorphism in **FOXE1** ($P=0.0028$) and three polymorphisms in **THRB** ($rs6792725: P=0.0087$, $rs13097208: P=0.0045$, $rs13066296: P=0.0056$), but failed to reach statistical significance after multiple-testing correction (Table 2).

For FT4, significant associations of 16 polymorphisms in seven genes were detected in the Rotterdam Study. Of these, two polymorphisms in **FOXE1** and one polymorphism in **FOXE1** remained significant at $P<0.0033$ in the meta-analysis of the four populations (Table 3). In addition, a suggestive association was detected for one polymorphism in **SLC16A10** ($P=0.0110$), but failed to reach statistical significance after multiple-testing correction (Table 3).

In a separate meta-analysis comprising only the three replication cohorts (i.e. Danish twin population, Scan Study, and Nijmegen Biomedical Study), all polymorphisms in **PDE8B** and **DIO1**, but not **FOXE1**, also showed significant associations with TSH (at $P<0.0022$) and FT4 (at $P<0.0033$; data not shown).

**Discussion**

In the present study, we studied the effects of genetic variation in 68 TH pathway genes on serum TSH and FT4 levels in 3777 subjects from four independent populations. Previously reported associations with genetic variation in **PDE8B**, **THRB** and **DIO1** were replicated. We demonstrate an effect of genetic variation in **FOXE1** on serum FT4 levels, and a borderline significant effect on serum TSH levels. In addition, a suggestive association of genetic variation in **SLC16A10** with serum FT4 levels was found.

Various genes have been studied in relation to serum TSH and TH levels in recent years, demonstrating that variants in the **PDE8B** (19, 29) and **DIO1** (8, 10–12) genes alter TSH and FT4 levels respectively, and suggesting a similar role for other genes such as **THRB** (19, 30) and **TSHR** (10, 13, 14).

**FOXE1**, also known as thyroid transcription factor 2 (TTF2), is a transcription factor in thyroid morphogenesis. Its importance is illustrated in mice with a homozygous inactivation of **FOXE1**, which exhibit a cleft palate and neonatal hypothyroidism due to an ectopic or absent thyroid gland (31). In humans, heterozygous missense mutations lead to neonatal hypothyroidism due to thyroid dysgenesis, cleft palate, chonial atresia, and spiky hair, which is referred to as the Bamforth–Lazarus syndrome (32). In our study, genetic variation in **FOXE1** was associated with FT4 levels, and a borderline significant association with TSH levels was found; e.g. in the Rotterdam Study, the per-allele effect was $-0.30$ pmol/l for FT4 and 0.11 mU/l for TSH. The lower FT4 and higher TSH levels in **FOXE1**-rs1443434 risk allele carriers suggest a moderately impaired thyroid development, since more TSH seems to be required to stimulate the thyroid to produce TH. It would therefore be interesting to study thyroid size and morphology in **FOXE1**-rs1443434 risk allele carriers using ultrasound.
Table 3 Effects of DIO1, FOXE1 and SLC16A10 polymorphisms on serum FT4 levels in the meta-analysis of four populations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Minor allele</th>
<th>( \beta ) (S.E.M.)*</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIO1</td>
<td>rs2235544</td>
<td>C</td>
<td>-0.16 (0.02)</td>
<td>8 \times 10^{-12}</td>
</tr>
<tr>
<td></td>
<td>rs11206244</td>
<td>A</td>
<td>0.16 (0.03)</td>
<td>5 \times 10^{-10}</td>
</tr>
<tr>
<td>FOXE1</td>
<td>rs1443434</td>
<td>C</td>
<td>-0.08 (0.02)</td>
<td>0.0003</td>
</tr>
<tr>
<td>SLC16A10</td>
<td>rs17606253</td>
<td>G</td>
<td>0.08 (0.03)</td>
<td>0.0110</td>
</tr>
</tbody>
</table>

*Effects were calculated using linear regression and expressed in s.d., corrected for age and gender.
**Reached the significant multiple-testing corrected \( P \) value (i.e. \( P < 0.0033 \)).

Various studies have identified the FOXE1 region as a susceptibility locus for thyroid cancer (33–35). A recent genome-wide association study identified a polymorphism (i.e. rs965513), which was associated with both thyroid cancer risk and lower serum TSH and T4 levels (33). As this polymorphism is located in an LD region with FOXE1 as the nearest gene (57 kb distance), the authors concluded that the effects of rs965513 might be mediated through processes involving FOXE1. Our data identified a genetic variant (i.e. rs1443434) in the FOXE1 gene itself that is associated with both serum FT4 and TSH levels, which is in moderate LD with the gene itself. A higher TSH level will be required to maintain normal levels of cAMP levels in response to TSH. Consequently, a higher TSH level might lead to increased PDE8B activity, resulting in lower cAMP levels in response to TSH. Hence, a higher TSH level will be required to maintain normal levels of TH. This hypothesis is supported by our findings, which show an association of genetic variation in PDE8B with TSH but not with FT4 levels. Similar to the results of Arnaud-Lopez et al., most associated polymorphisms in our study are located in intron 1 of the PDE8B gene, a region of high LD (see http://www.hapmap.org). PDE8B is also expressed in the adrenal gland and an inactivation mutation in PDE8B has been identified in a patient with micronodular adenocortical hyperplasia, leading to Cushing’s syndrome (37). No information was provided about the thyroid state of this patient.

The DIO1 gene encodes the iodothyronine deiodinase type 1 (D1). D1 is present in liver, kidney, and thyroid, and plays a key role in the production of the active hormone triiodothyronine (T3) from T4 and in the clearance of the metabolite rT3. DIO1-rs2235544 and DIO1-rs11206244 are in high LD (see http://www.hapmap.org). Associations of these polymorphisms with serum TH levels have been reported previously (8, 10–12, 20), and are replicated in our study.

The SLC16A10 gene, encoding for MCT10, is a transporter which facilitates both uptake and efflux of T3 and T4 (38). MCT10 has a wide tissue distribution, including intestine, kidney, liver, skeletal muscle, heart, and placenta (39). In the meta-analysis of the four populations, a polymorphism in MCT10 showed an association with altered serum FT4 levels. This association did not reach statistical significance after multiple-testing correction at \( P < 0.0033 \). Therefore, despite a low \( P \) value (rs17606253: \( P = 0.0110 \)), replication is needed in future studies. rs17606253 is located in intron 3 of the SLC16A10 gene, a region of high LD (http://www.hapmap.org). Exons 4 to 6 are also included in this region, coding for part of the transmembrane domain and the C-terminal domain. However, future studies need to clarify the exact functional variant in this region.

The structure of the SLC16A10 gene is highly homologous to that of the SLC16A2 (MCT8) gene (38). SLC16A2 is located on the X-chromosome, and was therefore not analyzed in this study. Among other tissues, MCT8 is highly expressed in brain, and mutations in MCT8 result in high levels of serum T3 and a syndrome of severe psychomotor retardation, known as the Allan–Herndon–Dudley syndrome (40). So far, no patients with mutations in MCT10 have been reported. Our results suggest that patients with mutations in MCT10 might be biochemically characterized by abnormal FT4 levels, in addition to other features.

We additionally found associations of genetic variation in the THRB gene (encoding TRβ1) with serum TSH levels. TH action is mediated via TRα and TRβ. Among other tissues, TRβ is expressed in liver and kidney and is the predominant receptor in the negative feedback regulation of the hypothalamus–pituitary–thymus axis. Mutations in TRβ lead to the TH resistance syndrome, which is biochemically characterized by increased levels of TH and a non-suppressed TSH. Common genetic variation in THRB has previously been reported to be associated with serum TSH levels (19, 30). However, results were inconsistent (30) or based on a single population (19). In this study, three polymorphisms in low LD and located in intron 1
resequencing efforts of these regions. The genes selected, which could be detected by large variation in serum TH levels. However, we cannot exclude potential effects of rare polymorphisms in the majority of these TH pathway genes. Based on our results, we conclude that high-frequency polymorphisms in one of its components. Based on our results, we conclude that high-frequency polymorphisms in the majority of these TH pathway genes do not play an important role in inter-individual variation in serum TH levels. However, we cannot exclude potential effects of rare polymorphisms in the genes selected, which could be detected by large resequencing efforts of these regions.

Strengths of our analyses include the large-scale approach with a high number of TH pathway genes studied in relation to both TSH and FT$_4$ levels, the high coverage of genetic variation in the studied genes and the use of multiple, large population-based cohorts. Our study also has some potential limitations. Promising polymorphisms were selected based on their effects in the Rotterdam Study. Study characteristics might influence these effects and may therefore interfere with the selection of promising hits. However, the Rotterdam Study is not a selection of the general population but a population-based cohort study. To exclude confounding by thyroid disease, we excluded subjects with known thyroid disease or thyroid medication usage. We therefore do not think that these biases have strongly influenced our results.

Finally, we studied polymorphisms with an MAF higher than 5%. We therefore cannot exclude potential effects of rare polymorphisms in the studied genes on TH serum levels. However, most polymorphisms in the human genome are located within LD blocks, which will be largely covered by our selection of studied polymorphisms. We were therefore powered to detect part of the effects of rare polymorphisms in the studied genes on TH serum levels.

In summary, we performed a large-scale candidate gene study of TH pathway genes for serum TSH and FT$_4$ levels, with replication in three independent populations. Previously reported associations with PDE8B, THRB, and DIO1 were replicated. We report a role for FOXE1 in inter-individual variation in serum FT$_4$ levels, and found a borderline significant association with serum TSH levels. In addition, a suggestive association of genetic variation in SLC16A10 with serum FT$_4$ levels was found.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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