Sample Collection and Handling

Blood samples were collected from patients who matched the study criteria by a specialized licensed nurse. Blood (5 mL) was withdrawn and distributed into anticoagulant – free plain tube (3 mL) and evacuated EDTA tube (2 mL). The blood sample in the plain tube was centrifuged after 30 minutes of sampling and serum was isolated and stored at -20°C and sent to Ibn Alnafees Hospital laboratory for biochemical analysis. Assays include TSH, fT3 and fT4. Assays were carried out using the AxSYM instrument (Abbott Laboratories, IL, U.S.A.) and commercially available diagnostic kits (Abbott Laboratories). Samples were assayed in duplicate and the mean of the paired results was determined. The blood collected in the EDTA tubes was used for DNA extraction.

Molecular Analysis

DNA Extraction

The venous blood, which was collected in the evacuated EDTA tubes, was used for DNA extraction that was performed using the Promega wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the standard protocol provided by the manufacturer.

PCR-RFLP

Reference sequence and details of SNPs, PCR primers’ design and restriction enzymes were obtained by searching the UCSC Genome Bioinformatics Site, Primer3 program and NEBcutter program, respectively.32-36 The location for the three SNPs, Rs939348 on the \textit{THRα} gene, and Rs2268458 and Rs2239610 on the \textit{TSHR} gene, is described in Table 1. The genomic DNA was amplified using the following steps: denaturation of double stranded genomic DNA at 94°C for 5 minutes, DNA amplification using 30 cycles. Each cycle consists of: denaturation at 94°C for 30s, annealing for 30s, extension at 72°C for 40s, final elongation at 72°C for 7 minutes, and ending reaction at 4°C. Table 1 summarizes restriction enzymes and restricted fragments for all SNPs. The restriction enzyme digestion was carried out in 20 µl containing 1 unit of enzyme and 10 µl of PCR product and incubated at 37°C for overnight. All enzymes were obtained from Fermentas (GmbH, St. Leon-Rot, Germany). PCR products and digested fragments were detected using electrophoresis on 2% agarose.

Statistical Analysis

Data was analyzed using the SPSS version 17 package (SPSS Inc, Chicago, USA) for Windows. Continuous variables were expressed as mean (standard deviation) and the differences were accomplished by comparison via student’s unpaired 2-sided t-test or one way ANOVA as appropriate. Discrete variables were expressed as counts and frequencies and were compared using the chi-square test. If N<5, exact Fisher statistic was used. The genotype distributions of SNPs were analyzed in agreement with the Hardy-Weinberg equilibrium. A significant difference is considered at P <0.05.

Results

The mean age of patients was 39.4 years, male to female ratio was 1:8 and the mean BMI (kg/m²) was 29.3. Of the 228 participants, 23.3% were dyslipidemic, 17.5% were diabetic and 23.2% were hypertensive (Table 2). The average duration of disease was 4.8 years. About 44% of the participants had a family history of thyroid disorders (Table 2).

Table 1. The PCR primers, restriction enzymes and sizes of the amplified and digested fragments of the examined SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>PCR product (bp)</th>
<th>Primers</th>
<th>Restriction enzyme</th>
<th>Restricted fragments (bp)</th>
</tr>
</thead>
</table>
| Rs939348 | 190          | F: 5’CCTGTGTCCTCCCAGCTTAGG ‘3  
R: 5’CCACCAGACTCACAGCCTCT ‘3 | MesI       | C allele 190  
T allele 48 & 142 |
| Rs2268458 | 162          | F: 5’CTAACCAAGCAGGAGGGGACAC’3  
R: 5’CCACTGCTAAAGGAGGAGCAT’3 | AluI       | T allele 162  
C allele 100 & 62 |
| Rs2239610 | 172          | F: 5’CCAGAGATCAAGGCGTCAGTCTG ‘3  
R: 5’CCAAGTGTGGGCGATTAAAGT ‘3 | NlaIV      | G allele 172  
C allele 142 & 30 |