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Rare and common genetic variations in the Keap1/Nrf2 antioxidant response pathway impact thyroglobulin gene expression and circulating levels, respectively

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ABSTRACT

Nuclear factor, erythroid 2-like 2 (Nrf2) is a transcription factor that has been gaining attention in the field of pharmacology and especially in the chemoprevention of diseases such as cancer, metabolic and neurodegenerative diseases, etc. This is because natural compounds such as sulforaphane, which is found in broccoli sprout extracts, can activate Nrf2. The repertoire of the roles of Nrf2 is ever increasing; besides its traditional antioxidant and cytoprotective effects, Nrf2 can have other functions as a transcription factor. We have recently shown that Nrf2 directly regulates the expression of thyroglobulin (Tg), which is the most abundant thyroidal protein and the precursor of thyroid hormones. Two functional binding sites for Nrf2 (antioxidant response elements, AREs) were identified in the regulatory region of the TG gene. Interestingly, we then observed that one of these AREs harbors a rare single-nucleotide polymorphism (SNP). Also recently, we performed the first genome-wide association study (GWAS) for common SNPs that impact the circulating levels of Tg. Based on these investigations, we were triggered (i) to investigate whether common SNPs in the Nrf2 pathway correlate with circulating Tg levels; and (ii) to examine whether the rare SNP in one of the TG regulatory AREs may affect gene expression. To address the first question, we analyzed GWAS data from a general population and its two subpopulations, one with thyroid disease and/or abnormal thyroid function tests and the other without, in which circulating Tg levels had been measured. Statistically significant associations with Tg levels were observed in the genes encoding Nrf2 and Keap1, including, notably, a known functional SNP in the promoter of the gene encoding Nrf2. Regarding the rare SNP (rs778940395) in the proximal ARE of the TG enhancer, luciferase reporter gene expression studies in PCCl3 rat thyroid follicular cells showed that this SNP abrogated the basal and sulforaphane- or TSH-induced luciferase activity, behaving as a complete loss-of-function mutation. Thus, both rare and common genetic variation in the Keap1/Nrf2 pathway can impact TG expression and Tg circulating levels, respectively.

1. Introduction

The transcription factor nuclear factor, erythroid 2-like 2 (Nfe2l2), also known as NFE2-related factor 2 (Nrf2), lies central to the regulation of a battery of antioxidant, cytoprotective and tissue-specific homeostatic genes [1]. Under basal conditions, Nrf2 is mainly sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1), a protein tethered to the actin cytoskeleton that serves as an adaptor facilitating the ubiquitination of Nrf2 by cullin 3 (Cul3); poly-ubiquitinated Nrf2 is subsequently degraded by the proteasome [2]. Keap1 is a protein rich in cysteine residues, whose sulphhydryl groups act as “sensors” for oxidative or electrophilic stimuli as they react with
For the SNPs that are within transcripts, both the mRNA (NM) and genomic (NC) references are given. Note that the minor allele always refers to the genomic reference (NC). G: general population; H: healthy population (without any history, sign, or laboratory indication of thyroid disease); D: subgroup with a history, sign, or laboratory abnormality indicative of thyroid disease* (i.e., thyroid medication, thyroideotomy, thyroid autoimmunity, or levels of TSH, free T4, free T3 and/or Tg outside the respective reference range). ALS: amyotrophic lateral sclerosis; COPD: chronic obstructive pulmonary disease; PD: Parkinson’s disease. * Effect of the indicated minor allele on Tg levels. ↑: increase; ↓: decrease. ** Known disease/phenotype associations of the respective SNP. All SNPs with at least one reported association are listed, but not all references are necessarily cited for each SNP).

<table>
<thead>
<tr>
<th>Gene/SNP</th>
<th>p-value</th>
<th>Tg*</th>
<th>Location</th>
<th>Functionality</th>
<th>Disease/Phenotype**</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFE2L2 rs76066649</td>
<td>G: 0.0197 H: 0.086 D: 0.131</td>
<td>↑, allele T</td>
<td>NC_000002.11.g.178130071C &gt; T</td>
<td>2 kb upstream variant</td>
<td>High cholesterol [31], ulcerative colitis [32], maternal acetaminophen and asthma [33], PD [34], ALS [35]</td>
</tr>
<tr>
<td>KEAP1 rs11668429</td>
<td>G: 0.086 H: 0.016 D: 0.737</td>
<td>↑, allele G</td>
<td>NC_000019.9g:10616303T &gt; G</td>
<td>promoter</td>
<td>Childhood asthma [36]</td>
</tr>
<tr>
<td>KEAP1 rs9676881</td>
<td>G: 0.173 H: 0.027 D: 0.864</td>
<td>↑, allele A</td>
<td>NC_000019.9g:10596780G &gt; A</td>
<td>0.5 kb downstream variant</td>
<td>COPD [37], diabetes [38]</td>
</tr>
<tr>
<td>KEAP1 rs1048290</td>
<td>G: 0.197 H: 0.03 D: 0.92</td>
<td>↑, allele C</td>
<td>NM_012289.3c:1413C &gt; G (p.Leu471 = )</td>
<td>exon 4 synonymous variant</td>
<td>Renal protection against cisplatin [39], temporal lobe epilepsy and drug-resistant epilepsy [40], diabetes [38], cognitive impairment [41], breast cancer [24]</td>
</tr>
</tbody>
</table>

The list of SNPs investigated in this study comprised all SNPs in the KEAP1, NFE2L2 and CUL3 genes that had been previously shown to be associated with other phenotypes or diseases. This list was compiled from a literature review, including both review papers of this topic [14,15], as well as original publications. These are cited in Tables 1 and 2; the references in these Tables are not exhaustive in terms of the associated diseases and phenotypes, because the criterion for inclusion of a SNP was the presence of at least one disease or phenotype. The dbSNP and ClinVar databases were also searched. Only germline SNPs associated with a disease or phenotype were included; somatic mutations were excluded.

The population that was checked for SNPs in the Nrf2 pathway comes from two Croatian cohorts as previously described [13] within the 10,001 Dalmatians project [16]. Analyses of the GWAS data were performed in three groups: (i) a population without thyroid disease and with normal thyroid function tests, comprising 1094 subjects; this is the population that has been previously published [13]; (ii) a population with thyroid disease and/or abnormal thyroid function tests, comprising 815 subjects; and (iii) the above two groups combined, comprising 1909 subjects and considered as a general population. The analyses of the GWAS data for the healthy subgroup have been previously described [13]; for the present work, the data from the general population and thyroid population were also analyzed, in the same manner as previously reported [13]. Thyroid disease and/or

**Table 1**

SNPs in **NFE2L2, KEAP1** and CUL3 associated with various human diseases or phenotypes and with Tg levels.

For the SNPs that are within transcripts, both the mRNA (NM) and genomic (NC) references are given. Note that the minor allele always refers to the genomic reference (NC). G: general population; H: healthy population (without any history, sign, or laboratory indication of thyroid disease); D: subgroup with a history, sign, or laboratory abnormality indicative of thyroid disease* (i.e., thyroid medication, thyroideotomy, thyroid autoimmunity, or levels of TSH, free T4, free T3 and/or Tg outside the respective reference range). ALS: amyotrophic lateral sclerosis; COPD: chronic obstructive pulmonary disease; PD: Parkinson’s disease. * Effect of the indicated minor allele on Tg levels. ↑: increase; ↓: decrease. ** Known disease/phenotype associations of the respective SNP. All SNPs with at least one reported association are listed, but not all references are necessarily cited for each SNP).
Table 2  
SNPs in NFE2L2, KEAP1 and CUL3 associated with various human diseases or phenotypes but not with Tg levels.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>p-value</th>
<th>Location</th>
<th>Functionality</th>
<th>Disease/Phenotype*</th>
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<tr>
<td>NFE2L2</td>
<td>rs7557529</td>
<td>G: 0.865</td>
<td>NC_000002.11:g.178127597C &gt; G</td>
<td>5’ region – 5238G &gt; A</td>
<td>PD [34], ALS [35], age-related cataract [42]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs35652124 (synonym)</td>
<td>G: 0.072</td>
<td>NC_000002.11:g.178126546G &gt; T</td>
<td>2 kb upstream variant – 214A &gt; G</td>
<td>Ulcerative colitis [32], lupus with nephritis [43], PD [44], gastric cancer and H. pylori infection [45,46], vitiligo [47], cardiovascular disease [48], vasodilation [49], ALS [35], olozoaosperrna [50]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs6721961 (synonym)</td>
<td>G: 0.59</td>
<td>NC_000002.11:g.178124687A &gt; G</td>
<td>2 kb upstream variant – 178A &gt; G, A &gt; C</td>
<td>PD [34], postmenopausal venous thromboembolism [51], breast cancer [24], acute lung injury [52-54], asthma [36], diabetes protection [55], blood pressure [31,48], BPD [56], H. pylori infection [45], vasodilation [49], vitiligo [47], semen quality in smokers [57], olozoaosperrna [50], cerebrovascular disease [31], lung adenocarcinoma [58]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs2886161</td>
<td>G: 0.0678</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], ALS [35], age-related cataract [42]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs36472322 (synonym)</td>
<td>G: 0.012</td>
<td>NC_000002.11:g.178124678T &gt; G</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs4234877</td>
<td>G: 0.0713</td>
<td>NC_000002.11:g.178127765C &gt; T</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
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<tr>
<td>NFE2L2</td>
<td>rs1300169</td>
<td>G: 0.971</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
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<tr>
<td>NFE2L2</td>
<td>rs1806649 (synonym)</td>
<td>G: 0.857</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
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<tr>
<td>NFE2L2</td>
<td>rs32987104 (synonym)</td>
<td>G: 0.647</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
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<tr>
<td>NFE2L2</td>
<td>rs3010350 (synonym)</td>
<td>G: 0.82</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs584410589 (synonym)</td>
<td>G: 0.647</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs1962368 (synonym)</td>
<td>G: 0.82</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs14013504 (synonym)</td>
<td>G: 0.82</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs1757417 (synonym)</td>
<td>G: 0.647</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs6083775 (synonym)</td>
<td>G: 0.647</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
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<tr>
<td>NFE2L2</td>
<td>rs13035806 (synonym)</td>
<td>G: 0.446</td>
<td>NC_000002.11:g.178127639T &gt; C</td>
<td>intron 1</td>
<td>PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [59], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]</td>
</tr>
<tr>
<td>Gene</td>
<td>SNP</td>
<td>p-value</td>
<td>Location</td>
<td>Functionality</td>
<td>Disease/Phenotype*</td>
</tr>
<tr>
<td>------</td>
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<tr>
<td>NFE2L2</td>
<td>rs10188193</td>
<td>G: 0.464, H: 0.292, D: 0.73</td>
<td>NC_000002.11:g.178120391 T &gt; C</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs10188107</td>
<td>G: 0.464, H: 0.292, D: 0.73</td>
<td>NC_000002.11:g.178120312 T &gt; G</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs10497511</td>
<td>G: 0.464, H: 0.292, D: 0.726</td>
<td>NC_000002.11:g.178119296 G &gt; C</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs2001297</td>
<td>G: 0.473, H: 0.308, D: 0.725</td>
<td>NC_000002.11:g.178118550 C &gt; A</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs10930781</td>
<td>G: 0.676, H: 0.266, D: 0.64</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs10575199</td>
<td>G: 0.676, H: 0.266, D: 0.64</td>
<td>NC_000002.11:g.178118550 C &gt; A</td>
<td>intron 1</td>
<td>Acute respiratory distress syndrome [52]</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>rs15534880</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>exon 1 missense variant</td>
<td>Immune deficiency, developmental delay, hypohomocysteinemia [67]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs11085735</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>5′ region &gt; 3306 T &gt; C</td>
<td>Breast cancer [24], renal protection against cisplatin [39]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs1048287</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>intron 3</td>
<td>Renal protection against cisplatin [39], FEV1 [37], breast cancer [24]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs8113472</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>intron 2</td>
<td>ALS [35], COPD [68]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs10412246</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>3′-UTR</td>
<td>Breast cancer survival [24]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs1177696</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>3′-UTR</td>
<td>Diabetes [38]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs7246953</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>3′-UTR</td>
<td>Diabetes [38], cognitive impairment [41]</td>
</tr>
<tr>
<td>KEAP1</td>
<td>rs11545829</td>
<td>ND</td>
<td>NC_000002.11:g.178114632 T &gt; G</td>
<td>3′-UTR</td>
<td>Esophageal squamous cell carcinoma [23]</td>
</tr>
</tbody>
</table>

For the SNPs that are within transcripts, both the mRNA (NM) and genomic (NC) references are given. Note that the minor allele always refers to the genomic reference (NC). G: general population; H: healthy subpopulation (without any history, sign, or laboratory indication of thyroid disease); D: subpopulation with a history, sign, or laboratory abnormality indicative of thyroid disease* (i.e., thyroid medication, thyroidectomy, thyroid autoimmunity, or levels of TSH, free T4, free T3 and/or Tg outside the respective reference range). ALS: amyotrophic lateral sclerosis; AMD: age-related macular degeneration; BPD: bronchopulmonary dysplasia; COPD: chronic obstructive pulmonary disease; PD: Parkinson’s disease; UTR: untranslated region. * Known disease/phenotype associations of the respective SNP. All SNPs with at least one reported association are listed in Tables 1 or 2, but not all references are necessarily cited for each SNPs.
abnormal thyroid function tests were defined as: thyroid medication use, history of thyroid surgery, history of thyroid disorders, or thyroid function test results out of the reference range (the parameters assessed were TSH, free T4, free T3, Tg, Tg autoantibodies and thyroid peroxidase autoantibodies). The study was approved by the Research Ethics Committees in Croatia and Scotland, and all subjects gave written informed consent. The analyses of the GWAS data for general population and the healthy subpopulation have been previously described; for the present work, the data from the thyroid population were also analyzed, in the same manner as previously reported [13].

2.2. Blood chemistries

Concentrations of thyroid hormones, Tg and autoantibodies were measured by immunoassay methods with the Liaison XL Biomedica Chemiluminescence Analyzer (DiaSorin, Saluggia, Italy) as previously described [13]. Measurements were performed in the Biochemistry Laboratory in the Department of Nuclear Medicine at the University Hospital of Split.

2.3. Plasmids construction

An already available luciferase reporter vector, hTGenh/prm-Luc [17], containing the human Tg upstream enhancer [18] and proximal promoter [19] cloned upstream of the luciferase reporter gene of the PGL3 vector (hereafter, pTg) was used to study the effect of the rs778940395 rare polymorphism. The pTg vector was used as a template to create a reporter bearing the rs778940395 using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) according to the manufacturer’s protocol. The following primers were used for the in vitro mutagenesis: pTg-rsF: 5′-CCCTGTGTCGCTGAAATCTTCTTGCTGGCCTGG-3′; pTg-rsR: 5′-CCAGGCCAGCAAGAAAGATTCCAGCACACAGG-3′. A previously created pTg enhancer/promoter construct bearing a mutant proximal ARE sequence (MUT) [9] was used as a positive control. All promoters constructs were verified by Sanger sequencing. In order to exclude any artifact arising from plasmid isolation of the WT and MUT constructs, two independent plasmid preparations (clones) were used in the experiments.

2.4. Cell culture

PCCL3 cells, a clonal rat thyroid follicular cell line [20], were cultured in Coon’s modified Ham’s F-12 under conditions described previously [9]. Culture media, supplements, sulforaphane and bovine TSH were all from Sigma-Aldrich (St. Louis, MO). Generation of a Nr2 knockout PCCL3 cell line using CRISPR/Cas9 technology has been previously described [9]. PCCL3 cells were transiently transfected with the different pTg reporter constructs in 96-well plates in complete medium using a 1:2 ratio of DNA and jetPRIME transfection reagent (Polyplus-transfection, Illkirch, France) respectively. The pEGFP-N1 plasmid (Clontech, Mountain View, CA) was included in all transfection experiments to monitor transfection efficiency and to normalize luciferase activities [21]. Treatment with sulforaphane (5 μM) or vehicle (DMSO) was performed 24 h after transfection. For TSH treatment, as soon as 24 h had passed after transfection, cells were starved for 48 h and then were treated with 0.5 ml/mL of TSH for 24 h. The starvation medium (4H medium) contained 0.2% FBS and four of the six hormones from the 4H medium [9] (TSH and insulin were excluded). Passive Lysis Buffer (Promega, Madison, WI) was used to lyse the cells 48 h after transfection and GFP fluorescence was measured using a NOVOstar multi-mode reader (BMG Labtech, Ortenberg, Germany) with excitation and emission wavelengths of 480 and 520 nm, respectively. The GFP fluorescence background of non-transfected cells was used to blank the fluorescence measurements. Then, luciferase activities were measured in the same plate using the Luciferase Assay System (Promega) according to the manufacturer’s protocol. Luciferase activities were normalized to GFP fluorescence and relative luciferase activities were expressed as fold change over control.

2.5. Statistics

Analyses of the GWAS data were performed as previously described in detail [13]. Briefly, Tg levels were adjusted for age and sex using linear regression analysis and then derived residuals were inverse normal-transformed and included in the linear mixed model, which accounts for population structure and relatedness. “SNPTEST” and R software were used to identify independently associated SNPs. Even through the data examined were derived from a GWAS, the present study took a hypothesis-based approach, wherein only a small set of SNPs was investigated; the statistical threshold applied was thus a p-value < 0.05.

In the cell-based studies, the normalized luciferase ratios were analyzed using the BootstrapRatio application (http://rht.iconcologia.net/stats/bRatio/index.html). This software tool is based on bootstrapping and resampling methods, without any assumption on the underlying probability distribution for the data analyzed [22]. The graphs were prepared using GraphPad Prism v7 (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. SNPs in KEAP1 and NFE2L2 genes are associated with circulating Tg levels

Several SNPs in NFE2L2 have been previously associated with various human diseases and/or phenotypes (Tables 1 and 2). Among them are especially three functional SNPs in the promoter of NFE2L2 itself (rs35652124, rs6706649 and rs6721961), which have shown to regulate its transcriptional activity with the minor alleles leading to reduced transcription [23]. Table 1 lists the SNPs with statistically significant p-values in the GWAS data in at least one population (general population, health subpopulation or thyroid disease subpopulation).

For NFE2L2, only rs6706649 was significantly associated with Tg levels, and only in the general population (p = 0.0197). The minor allele was associated with lower Tg levels. This is consistent with the fact that rs6706649 is one of the three functional SNPs in the promoter of NFE2L2 [20], and our previous finding that Nrf2 has a positive impact on the transcription of the gene encoding Tg and the thyroidal levels of Tg protein [9].

For KEAP1, three SNPs were significantly associated with Tg levels, all of them in the healthy population only (rs11668429, p = 0.016; rs9676881, p = 0.027; rs1048290, p = 0.03). For all three, the minor alleles were associated with higher Tg levels. Since it is not known whether each of these three specific SNPs affects KEAP1 expression, it is not possible to comment whether the observed directional effect (minor alleles associated with higher Tg levels) is compatible with the aforementioned positive effect of Nrf2 signaling on Tg [9]. Nevertheless, the fact all three minor alleles significantly affect Tg levels in the same direction (increase) is consistent with the fact that these three SNPs are in tight linkage disequilibrium with each other, as shown by the present data and previous work [24].

Only one SNP in the CUL3 gene has been previously reported to be associated with a human disease or phenotype (rs2396092, associated with esophageal squamous cell carcinoma [25]. This SNP was not associated with Tg levels in the present study (Table 2).

3.2. One of the functional Nrf2 binding sites (ARE) on Tg regulating region harbors a very rare sequence variant.

The two binding sequences through which Nrf2 upregulates Tg gene transcription, ARE1 and ARE2, are located 2.8 kb and 3.2 kb, respectively, upstream of the transcription initiation site [9]. According to the
information currently available in the Database of Single Nucleotide Polymorphisms (dbSNP build 152), ARE1 harbors a very rare sequence variant, rs778940395 (NC_000008.11:g.132864133 T > A; frequency: 1/30958 in GnomAD; 1/3854 in ALSPAC; 0/3708 in TWINSUK) (Fig. 1A). As there are no published studies on this variant, we investigated its functionality in cell-based reporter gene after introducing it in the pTG promoter/enhancer luciferase reporter construct using in vitro mutagenesis as described above (Fig. 1B) [9].

3.3. The rs778940395 variant in the TG distal enhancer is a completely loss-of-function in terms of transcription activity

PCCL3 rat thyroid follicular cells were transfected with different plasmids: two clones that carry the wild-type ARE1 sequence (WT1 and WT2, positive controls); two clones that carry the rs778940395 variant (rs1 and rs2); or a previously generated mutant ARE1 version that we have shown to be completely inactivating (MUT, positive control) [9] (Fig. 1B). Both rs1 and rs2 showed significantly reduced ARE-driven luciferase activity at baseline as compared to WT1 (which was itself not different from WT2); their activity levels were in fact not different from those of the MUT clone (Fig. 2A). These results indicate that the rs778940395 variant abolishes the basal transcriptional activity of ARE1. Treatment with sulforaphane significantly induced ARE-driven luciferase activity of the TG enhancer in the WT1 and WT2 clones, but this induction was blunted in the rs1 and rs2 clones, again to a similar extent as in the MUT clone (Fig. 2A). These results indicate that the rs778940395 variant also abolishes the sulforaphane-inducible transcriptional activity of ARE1. The same experiment was repeated in Nrf2 knockout PCCL3 cells engineered by CRISPR/Cas9 technology. In the absence of Nrf2, no differences were observed either under basal conditions or in response to sulforaphane treatment in any of the clones (Fig. 2B). These data indicate that all the observed differences in Fig. 2A are Nrf2-dependent; thus, the rs778940395 variant abolishes the Nrf2-dependent transcriptional activity of ARE1, both the basal one and the sulforaphane-inducible one.

We have shown previously that Nrf2 mediates the induction of the TG promoter not only by sulforaphane but also by TSH [9]. We therefore repeated the experiment in PCCL3 WT cells and treated them with TSH, as described in the Methods. As expected, TSH induced ~2.5-fold the ARE-driven luciferase activity in WT1 and WT2 (Fig. 2C). The rs1

Fig. 2. The rare variant rs778940395 in the proximal ARE of the TG distal enhancer leads to complete loss of basal and inducible transcriptional activity. A. Introduction of rs778940395 abrogates the basal and sulforaphane-induced ARE1-driven TG promoter/enhancer luciferase activity. PCCL3 wild-type (WT) cells were cultured in complete medium in 96-well plates and were transfected with different TG promoter/enhancer luciferase reporter constructs. Specifically, two clones with wild-type (WT1 and WT2) proximal AREs, two clones carrying rs778940395 in ARE1 (rs1, rs2) and a previously validated mutant ARE1 clone were used (MUT). After 24 h, cells were treated with 5 µM sulforaphane (SLF) or vehicle (< 0.1% dimethyl sulfoxide) as control (C); 24 h later, cells were lysed and luciferase activity was measured. Each column represents the mean ± SD of 7 independent experiments. *p < 0.05 vs. WT1_C; †p < 0.05 vs. WT1_SLF; **p < 0.05 vs. MUT_C; ‡p < 0.05 vs. MUT_SLF. B. Deletion of Nrf2 abrogates all changes in the presence of a TG-driven TG promoter/enhancer luciferase activity induced by ARE1 mutation or sulforaphane treatment. The same experiment illustrated in A was performed in Nrf2 knockout (Nrf2KO) PCCL3 cells developed by Crispr/Cas9 technology. In the absence of Nrf2, the activity of the TG promoter/enhancer is similar to the untreated condition (MUT). The third construct (MUT) carries an ARE1

Fig. 1. The distal enhancer of TG harbors a rare SNP in one of its functional Nrf2 binding sites (AREs). A. rs778940395, T > A is located in the core sequence of one of the two AREs in the TG enhancer. The core ARE sequence is indicated in grey. The nucleotide base that changes is indicated in red. B. Plasmids constructs that express luciferase under the control of the TG promoter/enhancer were used in the present study. The first construct carries the wild-type (WT) core proximal ARE sequence (ARE1). In the second construct, SNP rs778940395 was introduced in ARE1 by in vitro mutagenesis. The third construct (MUT) carries an ARE1

modified by in vitro mutagenesis that has been previously shown to be completely inactive. Binding sites for thyroid transcription factor 1 (TTF1) and a cAMP response element (CRE)-like potential binding site are also indicated.

A. Matana, et al. Biochemical Pharmacology xxx (xxxx) xxx–xxx
and rs2 clones showed significantly lower inducibility (~1.5-fold), which was again not different from that of the MUT clone (Fig. 2C). These results indicate that the rs778940395 variant also abolishes the TSH-inducible transcriptional activity of ARE1.

4. Discussion

Nrf2 is a proven druggable target for chemoprevention by natural antioxidants, and compounds like sulforaphane have been used in clinical trials in a variety of disease settings [26,27]. The emerging links between Nrf2 and the thyroid gland suggest that further research is warranted to better understand, and potentially also predict, whether pharmacological manipulation of Nrf2 can impact thyroid physiology or pathophysiology in a beneficial or detrimental manner, whether it is neutral, or whether genetic factors can act as determinants of this relationship. Indeed, human genetic studies indicate that potent activation of Nrf2 signaling throughout life can be detrimental for the thyroid; this conclusion is drawn from the fact that two published case reports on unrelated families from Japan have associated two different germ-line loss-of-functions mutations in KEAP1 with familial multinodular goiter inherited in an autosomal dominant manner [28,29]. However, these are very rare mutations, which can be actually considered “private”, since each of them has been documented only in a single family; thus, the relevance for the general population remains unknown.

To the best of our knowledge, the present study is the first one to explore the impact on the thyroid of inherited genetic variation in the Keap1/Nrf2 pathway by focusing on variants that are likely to lead to much more mild alterations of the pathway’s activity. The SNPs included in the GWAS analyses are classified as common variants, which, when associated with diseases or phenotypes, usually have small effect sizes. The results indicate that common genetic variation in NFE2L2 or KEAP1 is indeed associated with circulating Tg levels in the general population or in the healthy subpopulation, respectively. Regarding the molecular mechanism by which the SNPs affect Tg levels, because rs6706649 is known to be associated with lower NFE2L2 expression in vitro studies [23], we postulate that this leads to lower basal levels and lower activity of Nrf2, thereby causing decreased ARE-driven Tg gene transcription and ultimately decreased Tg circulating levels. We acknowledge that this is a hypothesis, as we do not have direct measures of NFE2L2 expression or Nrf2 activity in the thyroid gland of these subjects (this is a general limitation of association studies of the NFE2L2 functional SNPs). For the KEAP1 SNPs rs11668429, rs9676881 and rs1048290, we postulate that the observed effects on Tg levels are also likely mediated by altered Nrf2 activity; however, as noted in the Results, there are no published data on whether these three specific SNPs affect KEAP1 expression. No associations were found in the subpopulation with documented or suspected thyroid disease. One likely explanation is that thyroid disease per se or the existence of anti-Tg antibodies can induce substantial changes in the serum Tg levels or its measurement results, respectively, that obscure any mild impact of genetic variations in the Nrf2 pathway. Another explanation, not mutually exclusive with the aforementioned one, may be that mild impact of the common SNPs on Tg levels is not sufficient to cause thyroid disease. This is consistent with our recent clinical study that found no negative impact on thyroid hormonal or autoimmune status of a sulforaphane-containing beverage ingested over 12 weeks [12]. Taken together, these findings support the notion that, in contrast to potent and continuous activation of Nrf2 by loss-of-function mutations in KEAP1, mild or time-restricted alteration of Nrf2 pathway activity is more likely to be well tolerated by the thyroid.

Unlike the common SNPs interrogated in the GWAS, rs778940395 is very rare. Nevertheless, similar to the common SNPs, it can also be reasonably expected to have an overall mild effect at the molecular level. This is because, due to its rarity, it is highly unlikely to be encountered in the homozygous state. We hypothesize that rs778940395 abolishes the DNA binding of Nrf2, which we have previously shown to bind directly to the wild-type ARE1 [9]. Even though the mutant ARE1 has no transcriptional activity, a subject heterozygous for rs778940395 would still normally possess three functional AREs in his/her distal Tg enhancer, i.e., the ARE1 on the wild-type allele plus the two wild-type ARE2s. Hence, it can be estimated that rs778940395 would lower Tg mRNA levels by 25%. Even though ARE2 also regulates positively the basal and inducible activity of the TG enhancer [9], it does not harbor any SNPs, and thus the present analyses focused on the polymorphic ARE1.

One limitation of the present study is that, due to the rarity of rs778940395, we were not able to have access to carriers of this SNP in order to document whether they have a thyroid phenotype or not. Therefore, we believe that it is important to make these functional data publicly available, in order to motivate the identification and thyroidal phenotyping of carriers of this rare variant, possibly in the context of high-scale genotyping efforts like the 100’000 genomes project [30]. In the same spirit, we acknowledge that the “non-healthy” subpopulation analyzed in the present study was not homogeneous in terms of the underlying thyroid disease or thyroid function test abnormality. We therefore suggest that studies are thus warranted to examine the genetic association of SNPs in the Nrf2 pathway with specific thyroid diseases.

In conclusion, the present study demonstrates that both rare and common genetic variation in the Keap1/Nrf2 pathway can impact TG expression and Tg circulating levels, respectively, and it sets the stage for follow-up studies that will characterize associations of genetic variants in the pathway with specific thyroid diseases. Such studies are useful to predict the impact of pharmacological manipulation of Nrf2 on the thyroid and the underlying mechanisms, and they are complementary to clinical pharmacological studies with compounds that activate or inhibit Nrf2, of which the natural antioxidant sulforaphane serves as a prototype.

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References


