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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 (NFE2)-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); polyubiquitinated Nrf2 is subsequently degraded by the proteasome [2]. Keap1 is a protein rich in cysteine residues, whose sulfhydryl groups act as “sensors” for oxidative or electrophilic stimuli as they react with

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Table 1
SNPs in *NFE2L2*, *KEAP1* and *CUL3* associated with various human diseases or phenotypes and with Tg levels.

Gene	SNP	p-value	Tg*	Location	Functionality	Disease/Phenotype**
NFE2L2	rs6706649	**G: 0.0197** H: 0.086 D: 0.131	↓, allele T	NC_000002.11:g.178130071C > T	2 kb upstream variant	High cholesterol [31], ulcerative colitis [32] , maternal acetaminophen and asthma [33], PD [34], ALS [35]
KEAP1	rs11668429	G: 0.086 **H: 0.016** D: 0.737	↑, allele G	NC_000019.9:g.10616303 T > G	promoter	Childhood asthma [36]
KEAP1	rs9676881	G: 0.173 **H: 0.027** D: 0.864	↑, allele A	NC_000019.9:g.10596780G > A	0.5 kb downstream variant	COPD [37], diabetes [38]
KEAP1	rs1048290	G: 0.197 **H: 0.03** D: 0.92	↑, allele C	NC_000019.9:g.10600442G > C; NM_012289.3:c.1413C > G (p.Leu471 =)	exon 4 synonymous variant	Renal protection against cisplatin [39], temporal lobe epilepsy and drug-resistant epilepsy [40], diabetes [38], cognitive impairment [41], breast cancer [24]

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; 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 SNPs).

electrophilic or oxidative substances. These sulfhydryl groups also react with natural or synthetic activators of the Nrf2 pathway, including the so-called indirect antioxidants [3], leading to conformational changes in Keap1 that render it unable to present Nrf2 molecules to Cul3. Thus, *de novo* synthesized Nrf2 escapes Keap1/Cul3-mediated proteasomal degradation, and it accumulates the nucleus where it regulates (mainly, stimulates) the expression of its target genes by binding to antioxidant response element (ARE; 5′-NTGAG/CNNNGC-3′) sequences in their regulatory regions [4].

One of the many natural compounds that can activate the Keap1/Nrf2 pathway is sulforaphane (1-Isothiocyanato-4-(methylsulfinyl)butane). This indirect antioxidant belongs to the group of isothiocyanates and is derived mainly from cruciferous vegetables like broccoli sprouts. These vegetables actually contain glucoraphanin, the precursor of sulforaphane, which is converted to active sulforaphane by the enzyme myrosinase that is present in some plants as well as in the human intestinal microflora [5]. Sulforaphane has already been used in clinical trials with promising results in a variety of clinical settings (cancer, diabetes, skin disorders, heart disease, respiratory diseases, psychiatric diseases) [6]. It is believed that the beneficial effect of Nrf2 pathway activation by sulforaphane is not mediated only by the induction of an antioxidant/cytoprotective response but also through other pathways that Nrf2 can regulate in its capacity of transcription factor. For instance, in the case of diabetes, induction of Nrf2 pathway by sulforaphane can repress gluconeogenesis [7]. Hence, in view of the growing interest in antioxidants as Nrf2 pathway activators, research is also warranted not only to evaluate their effectiveness but also to identify the proper dosing regimens, and ideally also to predict and prevent adverse effects based on the known effects of Nrf2 on tissue-specific homeostasis [8]. In this context, we recently investigated the role of Nrf2 in thyroid homeostasis in mice and showed that genetic or pharmacological activation of Nrf2, besides regulating an antioxidant program in thyroid, can also directly activate the transcription of thyroglobulin (Tg), the precursor protein of thyroid hormones, by binding to two functional ARE sequences that are conserved between rodents and human and reside in the distal enhancer region of the *TG* gene [9]. Next, to address the issue of potential thyroidal toxicity of cruciferous vegetables preparations [10], we analyzed the serum of healthy volunteers who had ingested daily for 12 weeks a sulforaphane-rich broccoli sprout extract as part of a previous study [11]; we observed no adverse effect on thyroidal function, as assessed by thyroid function tests and thyroid autoantibody levels [12].

Based on these studies, we wondered whether genetic variation in the Nrf2 pathway among humans, in the form of either common single-nucleotide polymorphisms (SNPs) or rare mutations, could impact the expression of Tg and/or its circulating levels in the serum. Regarding common SNPs, we took advantage of the first genome-wide association study (GWAS) for Tg serum levels, which was performed in a Croatian population and was recently published [13]. Specifically, we examined whether SNPs in the *KEAP1, NFE2L2* and *CUL3* genes that had been previously shown to be associated with other phenotypes or diseases also had an impact on Tg levels. Regarding rare mutations, we focused on a very rare SNP (rs778940395) in the proximal functional ARE of the *TG* enhancer. The results identify common SNPs in the Nrf2 pathway that are associated with the circulating levels of Tg; they also demonstrate that the rare mutation in the *TG* completely inactivates its transcriptional activity.

2. Materials and methods

2.1. Study population and SNPs

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 subpopulation 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 2
SNPs in *NFE2L2*, *KEAP1* and *CUL3* associated with various human diseases or phenotypes but not with Tg levels.

Gene	SNP	p-value	Location	Functionality	Disease/Phenotype*
NFE2L2	rs7557529	G: 0.865 H: 0.42 D: 0.388	NC_000002.11:g.178135097C > T	5′ region − 5238G > A	PD [34], ALS [35], age-related cataract [42]
NFE2L2	rs35652124 rs57695243 (synonyms)	G: 0.072 H: 0.69 D: 0.073	NC_000002.11:g.178130073 T > C	2 kb upstream variant − 214A > G (previously − 653 or − 686)	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], oligoazoospermia [50]
NFE2L2	rs6721961 rs117801448 (synonyms)	G: 0.59 H: 0.484 D: 0.71	NC_000002.11:g.178130037 T > G/ NC_000002.11:g.178130037 T > C	2 kb upstream variant − 178A > G, A > C (previously − 617 or − 650)	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], vasodilatation [49], vitiligo [47], semen quality in smokers [57], oligoazoospermia [50], cerebrovascular disease [31], lung adenocarcinoma [58]
NFE2L2	rs2886161	G: 0.0678 H: 0.655 D: 0.0716	NC_000002.11:g.178127839 T > C	intron 1	PD [34], ALS [35]
NFE2L2	rs2364723	G: 0.0597 H: 0.63 D: 0.065	NC_000002.11:g.178126546G > T	intron 1	Basal and smoker FEV1 [37], FEV1 decline [59], cardiovascular disease [48], increased triglyceride levels [60]
NFE2L2	rs2364722	G: 0.0713 H: 0.72 D: 0.0596	NC_000002.11:g.178124787A > G	intron 1	Annual FEV1- decline [59,61], acute respiratory distress syndrome [52]
NFE2L2	rs4243387	G: 0.0713 H: 0.72 D: 0.059	NC_000002.11:g.178117765C > T	intron 1	Annual FEV1- decline [59,61], acute respiratory distress syndrome [52]
NFE2L2	rs13001694	G: 0.871 H: 0.241 D: 0.468	NC_000002.11:g.178118990A > G	intron 1	Basal and smoker FEV1 [37]
NFE2L2	rs1806649 rs58745895 (synonyms)	G: 0.857 H: 0.164 D: 0.191	NC_000002.11:g.178118152C > T	intron 1	PD [34], particulate matter and asthma/COPD hospital admission [62], COPD [60], asthma [36], coronary heart disease [63], ALS [35], renal cisplatin protection [39], increased triglyceride levels [60], reduced COPD mortality [60], FEV1 reduction [37]
NFE2L2	rs1962142 rs58448508 (synonyms)	G: 0.647 H: 0.22 D: 0.65	NC_000002.11:g.178113484A > T/NC_000002.11:g.178113484A > G	intron 1	Annual FEV1 decline [61], acute respiratory distress syndrome [52], cerebrovascular disease [31]
NFE2L2	rs6726395 rs57309289 (synonyms)	G: 0.71 H: 0.36 D: 0.94	NC_000002.11:g.178103229A > G	intron 1	Smoking-related FEV1 decline [37,59,61] and annual FEV1 decline in lung cancer [37], cholangiocarcinoma [64], increased triglyceride levels [60], reduced cardiovascular mortality [60], AMD [65]
NFE2L2	rs2001350 rs17515179 rs60883775 (synonyms)	G: 0.82 H: 0.563 D: 0.68	NC_000002.11:g.178100425C > T	intron 1	Annual FEV1 decline [61] , PD [34], ALS [35], acute respiratory distress syndrome [52]
NFE2L2	rs10183914 rs58731187 rs61374844 (synonyms)	G: 0.483 H: 0.13 D: 0.71	NC_000002.11:g.178097666C > T	intron 3	PD [34]
NFE2L2	rs2706110	G: 0.996 H: 0.712 D: 0.58	NC_000002.11:g.178092162 T > C	3′ region	Breast cancer [24], temporal lobe epilepsy [40], PD and pesticides exposure [66], cerebrovascular disease [31]
NFE2L2	rs13035806	G: 0.446 H: 0.95 D: 0.42	NC_000002.11:g.178091822G > A	3′ region	Age-related cataract [42]

(*continued on next page*)

Table 2 (*continued*)

Gene	SNP	p-value	Location	Functionality	Disease/Phenotype*
NFE2L2	rs10188193	G: 0.464 H: 0.292 D: 0.73	NC_000002.11:g.178120391 T > C	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs10188107	G: 0.464 H: 0.292 D: 0.73	NC_000002.11:g.178120312 T > G	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs10497511	G: 0.464 H: 0.292 D: 0.726	NC_000002.11:g.178119296G > C	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs2001297	G: 0.473 H: 0.308 D: 0.725	NC_000002.11:g.178118550C > A	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs10930781	G: 0.676 H: 0.266 D: 0.64	NC_000002.11:g.178114632A > T/NC_000002.11:g.178114632A > G	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs2364720	G: 0.696 H: 0.322 D: 0.68	NC_000002.11:g.178105144A > G	intron 1	Acute respiratory distress syndrome [52]
NFE2L2	rs1553488015	ND	NC_000002.11:g.178098954C > T; NM_006164.4(NFE2L2):c.91G > A (p.Gly31Arg)	exon 1 missense variant	Immunodeficiency, developmental delay, hypohomocysteinemia [67]
NFE2L2	rs1057519922	ND	NC_000002.11:g.178098810C > T; NM_001313904.1(NFE2L2):c.6G > A (p.Lys2 =)	exon 1 synonymous variant	Immunodeficiency, developmental delay, hypohomocysteinemia [67]
NFE2L2	rs1553487947	ND	NC_000002.11:g.178098806G > T; NM_006164.4(NFE2L2):c.239C > A (p.Thr80Lys)	exon 1 missense variant	Immunodeficiency, developmental delay, hypohomocysteinemia [67]
NFE2L2	rs1553487942	ND	NC_000002.11:g.178098804C > T; NM_001313904.1(NFE2L2):c.12G > A (p.Gln4 =)	exon 1 synonymous variant	Immunodeficiency, developmental delay, hypohomocysteinemia [67]
NFE2L2	rs2886162	ND	NC_000002.11:g.178133165A > G	5′ region − 3306 T > C	Breast cancer [24], renal protection against cisplatin [39]
KEAP1	rs11085735	G: 0.58 H: 0.12 D: 0.53	NC_000019.9:g.10602180A > C	intron 3	Renal protection against cisplatin [39], FEV1 [37], breast cancer [24]
KEAP1	rs1048287	G: 0.697 H: 0.64 D: 0.785	NC_000019.9:g.10610236A > G; NM_012289.3:c.474 T > C (p.Gly158 =)	exon 2 synonymous variant	ALS [35], COPD [68]
KEAP1	rs8113472	G: 0.72 H: 0.75 D: 0.732	NC_000019.9:g.10608064C > T/NC_000019.9:g.10608064C > A	intron 2	Breast cancer survival [24]
KEAP1	rs10412246	G: 0.848 H: 0.653 D: 0.95	NC_000019.9:g.10613191A > G	intron 1	Diabetes [38]
KEAP1	rs3177696	G: 0.652 H: 0.183 D: 0.66	NC_000019.9:g.10596811 T > C	3′-UTR	Diabetes [38]
KEAP1	rs7246953	G: 0.738 H: 0.261 D: 0.78	NC_000019.9:g.10621108G > C/NC_000019.9:g.10621108G > A	promoter	Childhood asthma [36]
KEAP1	rs11545829	ND	NC_000019.9:g.10599965G > A; NM_012289.3:c.1611C > T (p.Tyr537 =)	exon 5 synonymous variant	Diabetes [38], cognitive impairment [41]
CUL3	rs2396092	G: 0.737 H: 0.853 D: 0.38	NC_000002.11:g.225335290A > G	3′-UTR	Esophageal squamous cell carcinoma [25]

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 tests levels 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-rs_F: 5′-CCTGTGTGCTGAATCT TTCTTGCTGGCCTGG-3′; pTG-rs_R: 5′-CCAGGCCAGCAAGAAAGATTC AGCACACAGG-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 Nrf2 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 mIU/mL of TSH for 24 h. The starvation medium (4H medium) contained 0.2% FBS and four of the six hormones from the 6H 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 BootstRatio application (http://rht.iconcologia.net/stats/br/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 been 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 *NFE2L2* promoter [23], 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

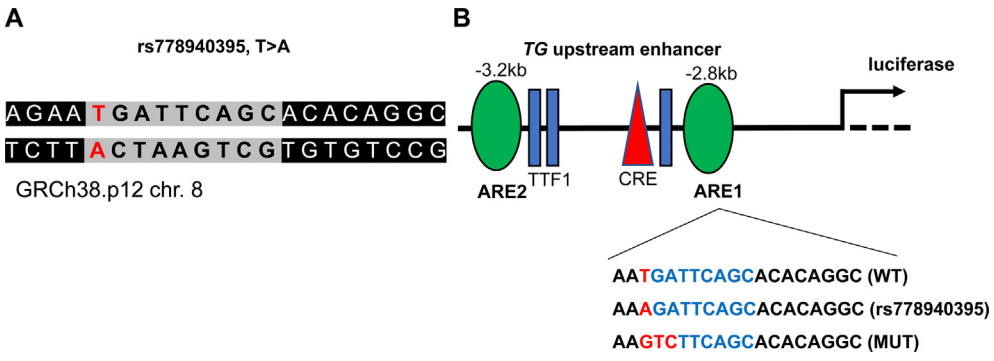

Fig. 1. The distal enhancer of *TG* harbors a rare SNP in one of its functional Nrf2 binding sites (AREs). A. rs778940395, T > A is located in the core sequence of one of the two AREs in the *TG* enhancer. The core ARE sequence is indicated in grey. The nucleotide base that changes is indicated in red. B. Plasmid constructs that express luciferase under the control of the *TG* promoter/enhancer 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.

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 r2 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. 2**A**). 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. 2**A**). 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. 2**B**). These data indicate that all the observed differences in Fig. 2**A** 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. 2**C**). The rs1

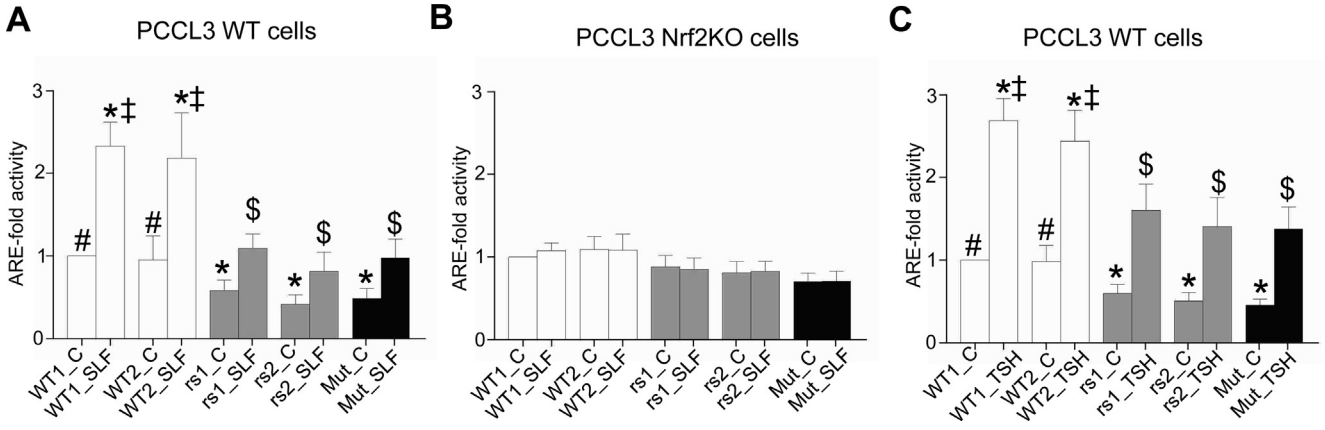

Fig. 2. The rare variant rs778940395 in the proximal ARE of the *TG* distal enhancer leads to complete loss of basal and inducible transcriptional activity. A. Introduction of rs778940395 abrogates the basal and sulforaphane-induced ARE1-driven *TG* promoter/enhancer luciferase activity. PCCL3 wild-type (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; $^{\ddagger}p < 0.05$ vs. MUT_SLF. B. Deletion of Nrf2 abrogates all changes in the ARE-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 unaffected by the presence or absence of rs77894039 as well as by treatment with sulforaphane. Each column represents the mean ± SD of 7 independent experiments. C. Introduction of rs778940395 to the *TG* promoter/enhancer blunts its TSH-induced activity, behaving like the complete loss-of-function mutant ARE version. 24 h after transfection, WT PCCL3 cells were starved in 4H medium (medium without TSH and insulin) supplemented with 0.2% FBS for 48 h, and then they were treated or not with TSH (0.5 mU/ml) for 24 h. Each column represents the mean ± SD of 7 independent experiments. *$p < 0.05$ vs. WT1_C; $^{\$}p < 0.05$ vs. WT1_TSH, $^{\#}p < 0.05$ vs. MUT_C, $^{\ddagger}p < 0.05$ vs. MUT_TSH.

and rs2 clones showed significantly lower inducibility (~1.5-fold), which was again not different from that of the MUT clone (Fig. 2**C**). 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 germline 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 *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 SNPs in order to document whether they have a thyroidal 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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