A promoter polymorphism in human interleukin-32 modulates its expression and influences the risk and the outcome of epithelial cell–derived thyroid carcinoma

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Interleukin (IL)-32 is an intracellular proinflammatory mediator that strongly modulates the inflammatory reaction. Recent studies have suggested the involvement of IL-32 in the pathogenesis of malignancies. We aimed to assess whether a known germ-line polymorphism in the IL32 promoter modulates IL-32 expression, and whether it influences susceptibility and/or outcome of epithelial cell–derived thyroid carcinoma (TC). In this study, IL32 genotype was assessed in 139 TC patients and 138 healthy controls and was correlated with TC susceptibility and clinical outcome. Furthermore, IL-32 messenger RNA expression and protein were assessed in TC tissues and functional consequences of genetic variants of IL32 were studied in a model of human primary immune cells. Results demonstrate substantial IL-32 expression in TC tumor tissue. Lipopolysaccharide (LPS) stimulation of primary immune cells revealed 2-fold higher expression of IL-32γ, but not IL-32β, in cells homozygous for the ancient T allele. Furthermore, production of LPS-induced cytokines was increased in cells bearing this T allele. Genetic analysis revealed that the ancient T allele was overrepresented in TC patients with odds ratio (95% confidence interval) = 1.71 (1.06–2.75). In addition, the cumulative radioactive iodine (RAI) dose received after total thyroidectomy was significantly higher in TC patients bearing the ancient T allele. In conclusion, individuals bearing genetic variants of IL32 that lead to an increased IL-32γ gene expression and higher production of proinflammatory cytokines have higher risk for developing epithelial cell–derived TC. Subsequently, they require higher dosages of RAI to achieve successful tumor remission. These data suggest an important role of IL-32 in the pathogenesis of TC.

Introduction

Inflammation and cancer are heavily intertwined. Both pro- and anti-tumorigenic effects are exerted by tumor-associated accessory cells, comprising neutrophils, macrophages and T cells that infiltrate the tumor microenvironment, which includes tumors that occur within the thyroid (1,2). Thyroid carcinoma (TC) is the most common endocrine malignancy (3). The vast majority of TCs is non-medullary TC that are derived from follicular epithelial cells (4,5). Primary treatment of patients with TC consists of total thyroidectomy with or without neck dissections (depending on the presence of lymph node metastases) followed by ablation of remnant thyroid tissue with radioactive iodine (RAI). Although the majority of patients reach remission, some require additional RAI or surgery because of persistent or recurrent disease. Initial studies have suggested an important role for inflammation in the occurrence and prognosis of TC (6,7). However, the mechanisms and cell types involved remain largely elusive.

Interleukin (IL)-32 has emerged as an intracellular pluripotent inflammatory mediator that is involved in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (8,9) and chronic obstructive pulmonary disease (10). In addition, IL-32 has also been implicated in neoplastic disorders including chronic myelomonocytic leukemia (11) and in the carcinogenesis of several solid tumors (12–14). On the functional level, overexpression of endogenous IL-32, especially the most potent IL-32γ isoform and to a lesser extent the IL-32β isoform, has been reported to amplify inflammatory signaling induced by tumor necrosis factor α (TNFα) (15) and to synergize with intracellular pattern recognition receptors for the production of several proinflammatory cytokines, including IL-1β and IL-6 (16,17). Through these mechanisms, IL-32 polarizes innate immune cells to induce Th1 and Th17 cellular responses (18). However, the molecular pathways involved in these effects remain obscure, although there are some indications that IL-32 could inhibit messenger RNA (mRNA) decay of proinflammatory cytokines (15) and could influence activation of transcription factors (19).

Genetic variation in IL32 has recently been associated with acute lung injury elicited in the context of pulmonary infection (20). Despite the suggested role of IL-32 in modulating inflammation and the development of malignant processes, little has been done to assess the role of IL32 gene polymorphisms for susceptibility to cancer in general, and to TC in particular. In this study, we hypothesized that genetic variation in IL32 influences the susceptibility to TC and potentially the clinical outcome of TC patients. At first, we have determined IL-32 mRNA and protein expression in paraffin-embedded TC tissue samples derived from papillary and follicular tumors. Additionally, functional consequences of the IL32 rs28372698 polymorphism for IL-32 mRNA expression and function were investigated. Furthermore, by analyzing a cohort of 139 TC patients and 138 healthy controls that were genotyped for this rs28372698 T/A genetic variant located in the promoter region of IL32, its potential association with TC susceptibility and/or clinical outcome was assessed.

Materials and methods

TC patients

All patients with histologically confirmed non-medullary epithelial cell–derived TC who visited the outpatient clinic at the Division of Endocrinology of the Department of Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands were asked to provide blood for genetic testing. The recruitment of the patients took place between November 2009 and June 2010. Primary treatment of the patients consisted of total or near-total thyroidectomy in all of the patients, and modified radical neck dissections in patients with confirmed nodal metastases. This was followed by ablation with RAI (131I) of residual thyroid tissue 4–6 weeks after surgery. If necessary, patients were treated multiple times with RAI to reach remission. Initial cure was defined as undetectable thyroid-stimulating hormone-stimulated thyroglobulin (Tg) in the absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastasis on the whole-body iodine scans and/or neck ultrasonographic examinations at 6 months after RAI ablation. Recurrence was defined as new evidence of loco-regional disease or distant metastasis >6 months after successful primary therapy. Current disease status was defined as in remission in case of undetectable Tg in the

Abbreviations: Ct, comparative threshold cycle; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger RNA; NIS, sodium iodide symporter; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RAI, radioactive iodine; RT, room temperature; SNP, single nucleotide polymorphism; TC, thyroid carcinoma; Tg, thyroglobulin; TNF, tumor necrosis factor α.

These authors share senior authorship.
absence of anti-Tg antibodies and no evidence of loco-regional disease or distant metastases at the last follow-up visit. Persistent disease was defined as detectable Tg and/or evidence of loco-regional disease or distant metastases.

Demographic and clinical characteristics, information about tumor treatment and follow-up were obtained from the patients’ medical records. Tumor-related parameters included tumor histology and TNM stage at diagnosis. Furthermore, information on the number of RAI therapy sessions, cumulative RAI dose, number of re-operations and external beam radiation therapy, if applicable, were retrieved from the patient’s medical records (Table I). No significant differences were observed between patients with different IL32 genotypes and clinical characteristics including gender, age and tumor histology.

The Dutch population-based control group consisted of 138 healthy controls (48% women, mean age 61 + 10 [standard deviation] years) having no evidence of thyroid cancer or other malignancies.

Ethical approval

This study was approved by the ethical committee of Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. All subjects gave written informed consent. The study has been performed in accordance with the Declaration of Helsinki.

Immunohistochemical staining of IL-32 in TC tissue

In specimens obtained from surgery, IL-32 protein expression was evaluated by immunohistochemical staining of paraffin-embedded TC tissue sections. To remove the paraffin, tissues were incubated twice in xylene and successively in 100, 96 and 70% of alcohol for 5 min each step. Antigens were retrieved with citrate buffer for 2 min in the microwave (800 W) and 10 min at room temperature (RT); citrate buffer: pH = 6.0, 16.4 ml sodium citrate [0.1 M] with 3.6 ml citric acid [0.1 M] in 180 ml H2O. The endogenous peroxidase activity was blocked with 3% of H2O2 in methanol for 15 min at RT. Furthermore, because tumor-like tissues contain endogenous biotin, this was blocked in the tissue sections by avidin/biotin blocking kit according to the manufacturers’ protocol (Vector Laboratories, Burlingame, CA). Sections were incubated with 20% goat serum diluted in phosphate-buffered saline (PBS) for 10 min and subsequently, with the first antibody (polyclonal goat-antihuman IL-32 AF53040 antigen or goat polyclonal IgG isotype control AB-108-C; R&D Systems, Minneapolis, MN), both 2.5 μg/ml in PBS supplemented with 5% goat serum, overnight at RT. After washing with PBS, sections were incubated with the second antibody (rabbit antigiot-BIOT-Vecto BA-5000), 1:500 diluted in PBS supplemented with 5% rabbit serum, for 30 min at RT. The ABC–horseradish peroxidase complex (ABCkit-HRP Vector PK-6101), 1:200 diluted in PBS, was applied to the sections for 30 min at RT. The substrate solution was added for 5 min at RT: 0.5 ml of diaminobenzidene in 9.5 ml of PBS and 10 μl of H2O2. Tissues were counterstained with hematoxylin for 30 s at RT. Slides were dehydrated with consecutive incubation in 70, 96 and 100% of alcohol and xylene (two times) for 5 s each step. Sections were mounted in Permount.

Genotyping for IL32 rs28372698 polymorphism

Venous blood was drawn from the cubital vein of all participants into 10 ml ethylenediaminetetraacetic acid tubes (Monoject). The mononuclear cell fraction was obtained by density centrifugation of diluted blood 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech, Harriburg, PA). Cells were washed twice in saline and suspended in RPMI (Invitrogen, Carlsbad, CA) supplemented with gentamicin (10 μg/ml), l-glutamine (10 mM) and pyruvate (10 mM). Cells were counted in a Coulter counter (Coulter Electronics, Indianapolis, IN) and the number was adjusted to 5 × 10^6 cells/ml. A total of 5 × 10^5 mononuclear cells in a 100 μl volume was added to round-bottom 96-wells plates (Greiner Bio-one, Monroe, NC) and incubated with either 100 μl of culture medium (negative control) or Escherichia coli lipopolysaccharide (LPS; 10 ng/ml; Sigma, St Louis, MO). All cytokines were measured after 24 h of incubation, using commercial enzyme-linked immunosorbent assay kits from R&D Systems for TNFα and IL-1β or from Sanquin, Amsterdam, The Netherlands for IL-6 and IL-10.

Real-time PCR

Peripheral blood mononuclear cells (PBMCs) stimulated for 24 h at 37°C were treated with TRZol reagent (Invitrogen) and total RNA purification was performed according to manufacturers’ instructions. RNA was isolated from paraffin-embedded tissue by lysis and RNA precipitation protocols. Exclusively neoplastic parts of the sections were selected for RNA isolation to specifically study tumor tissue. In brief, tissue samples were disrupted and homogenized by an overnight incubation with Proteinase K (Qiagen, Valencia, CA). RNA extraction was performed by using RNA-Bee according to the manufacturers’ protocol (AMS Biotechnology, Abingdon, UK), including chloroform phase separation and isopropanol precipitation. Isolated RNA was subsequently transcribed into complementary DNA by using random hexamers (Promega, Leiden, The Netherlands) followed by quantitative PCR using the SYBR Green method (Applied Biosystems). The following primers were used for detection of total IL-32: forward 5’-AGG-ACG-TGG-ACA-GGT-GAT-GAT-GTCT-3’ and reverse 5’-GTG-CCG-TAG-CCT-CTG-TCT-TTG-A-3’; IL-32α: forward 5’-CAG-TGG-AGG-ACG-TGG-GTC-ATC-TCA-3′ and reverse 5’-GGG-CCT-TCA-GCT-TCT-TCA-3′; and IL-32β: forward 5’-AGG-CCC-GAA-TGG-TAA-TGC-T-3’ and reverse 5’-CCA-CAG-TCT-GCT-CAG-TCT-C-3′. Data were corrected for expression of the housekeeping gene β2 microglobulin, for which the primers forward 5’-AGT-AGT-ATG-CCT-GGC-CTT-GTCT-3’ and reverse 5’-CCA-AAAT-GCG-GCA-TCT-TCA-3’ were used. Gene expression values were calculated by employing the comparative threshold cycle (Ct) method. The Ct data for IL-32 (isofoms) and the housekeeping gene β2-microglobulin were used to create ΔCt values (ΔCt = Ct target gene – Ct housekeeping gene). Therefore, the relative quantity was calculated by 2^-ΔCt.

<table>
<thead>
<tr>
<th>Table I. Clinical, pathological and treatment characteristics of TC patients</th>
<th>Variable</th>
<th>AA</th>
<th>TA</th>
<th>TT</th>
<th>Total</th>
<th>P-value*</th>
</tr>
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<tbody>
<tr>
<td>Patients (n)</td>
<td>46</td>
<td>72</td>
<td>21</td>
<td>139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>38/8</td>
<td>50/22</td>
<td>16/5</td>
<td>104/35</td>
<td>0.272</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis, years, (mean ± SD)</td>
<td>39.8 (±12.5)</td>
<td>38.5 (±13.5)</td>
<td>38.3 (±11.4)</td>
<td>38.9 (±12.8)</td>
<td>0.812</td>
<td></td>
</tr>
<tr>
<td>Tumor histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.457</td>
<td></td>
</tr>
<tr>
<td>PTC</td>
<td>29</td>
<td>57</td>
<td>13</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTC</td>
<td>14</td>
<td>7</td>
<td>12</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both PTC + FTC</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTC</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Re-operations</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean duration follow-up, months (mean ± SD)</td>
<td>125 (±106)</td>
<td>132 (±109)</td>
<td>134 (±136)</td>
<td>128 (±112)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DTC, differentiated TC; FTC, follicular TC; PTC, papillary TC, not further specified; F, female; M, male; SD, standard deviation.

*P-value of mean age at diagnosis and sex difference were calculated by one-way analysis of variance; P-value of tumor histology by Pearson χ² analysis.

Since diagnosis of TC (primary surgery).
Statistical analysis
The difference in genotype frequencies between the patients and the control group was analyzed in a gene dosage dependent, a dominant and a recessive model using logistic regression. The effect of the genotypes on epithelial-derived TC susceptibility was estimated by calculating odds ratios and their 95% confidence intervals using the same statistical methods. We also performed χ² analysis to determine whether tumor size, cumulative RAI dose, number of RAI treatments, disease status after thyroidectomy plus radio-ablation (if applicable) and current disease status were associated with the IL32 genotype. The following parameters were analyzed: (i) the tumor size at time of diagnosis was classified according to the sixth edition of the UICC TNM classification (21); (ii) the number of RAI treatments (including RAI ablation) as 0–1 treatments (e.g. no RAI ablation or exclusively ablation of thyroid rests after (near) total thyroidectomy) or ≥2 treatments; (iii) the cumulative RAI dosage as 0–3.7 GBq (0–100 mCi), 3.8–7.4 GBq (101–200 mCi) or >7.4 GBq (>200 mCi); (iv) the disease status after ablation as remission or persistent and (v) the current disease status as remission, persistent or recurrent (after previously documented remission).

To test for differences between the three different genotype groups (homozygous wild-type [ancient], heterozygous, homozygous mutant [derived]) in mean age at diagnosis, sex distribution or tumor histology (potential confounders), one-way analysis of variance and Pearson χ² analysis were used when appropriate. Differences in mRNA expression and cytokine production capacity of cells were statistically assessed by the Mann-Whitney U test.

All statistical analyses were carried out with the SPSS software package (version 16.0). Overall, statistical tests were two-sided and a P < 0.05 was considered statistically significant.

Results

Immunohistochemical staining of IL-32 on TC tissue sections
TC tissue obtained from patients who underwent thyroid surgery were stained for IL-32 protein expression. The staining revealed intracellular expression of IL-32 in thyroid epithelial cells, endothelial cells of blood vessels and in immune cells present in infiltrates (Figure 1A), indicating that IL-32 is present in thyroid tumor. Of note, for the immunohistochemical stainings, it was necessary to block endogenous biotin activity, as some of our initial stainings including negative control and isotype conditions were positively stained. By blocking endogenous biotin, aspecific staining of the sections was prevented effectively.

IL-32 mRNA expression in TC tissue sections
TC tissue sections obtained from patients who underwent thyroid surgery were used to extract total RNA and to measure IL-32 mRNA expression, total and the active IL-32 isoforms IL-32β and IL-32γ separately. Tissues were derived from patients suffering from either papillary or follicular TC. The analysis revealed the presence of IL-32 mRNA in the tumor tissue, which was true for both the IL-32β and IL-32γ isoform (Figure 1B). Furthermore, these data show that IL-32 was highly expressed in 15% of patients, moderately expressed in 41% of patients and had a low expression in 44% of the patients. These results indicate that bioactive isoforms of IL-32 are present in TC tissue and may be involved in thyroid pathology.

IL32 rs28372698 promoter polymorphism: effect on IL-32 expression and LPS-induced cytokines
In order to assess whether the IL32 rs28372698 polymorphism influences IL-32 mRNA expression, PBMCs from healthy donors that were either homozygous bearing the ancient T allele or homozygous for the derived A allele were cultured for 24 h with or without the TLR4 ligand LPS, a standard stimulus for the production of proinflammatory cytokines. Subsequently, IL-32 mRNA expression was measured, after which a statistically significant 2-fold difference in IL-32 expression after LPS stimulation was observed between the cells isolated from individuals with the different genotypes (Figure 2A). In more detail, cells homozygous for the ancient T allele exhibited increased

Fig. 1. IL-32 expression in TC. (A) Representative immunohistochemical staining on TC tissue sections for IL-32 and isotype control (×400 magnification). (B) Relative mRNA expression of total IL-32 and the separate isoforms IL-32β and IL-32γ in TC tissue sections, both derived from papillary and follicular tumor material (N = 27). The horizontal line indicates the mean value.
expression compared with the unstimulated condition, whereas in cells homozygous for the derived A allele, IL-32 expression was decreased compared with baseline. No differences were apparent between the genotypes in the unstimulated conditions. Transcriptional analyses specifically for the IL-32 isoforms IL-32β and IL-32γ revealed that the difference in total IL-32 expression between the genotypes is the result of a fairly identical difference in expression of the IL-32γ isoform. Again, increased expression was detected in the individuals homozygous for the T allele upon stimulation with LPS, as compared with the same condition with cells homozygous for the A allele.

Furthermore, cytokine measurements in the supernatants of cells stimulated for 24 h revealed significantly higher production of TNFα, IL-1β, IL-6 and IL-10 in cells homozygous for the ancient T allele as compared with cells homozygous for the derived A allele (Figure 2B). These results suggest that IL-32γ expression and production of inflammatory cytokines are linked and demonstrate that the IL-32 rs28372698 polymorphism has functional consequences for the inflammatory reaction.

Genetic association of IL-32 polymorphism with susceptibility to TC
The observation that IL-32 is present in TC tissue and that the rs28372698 genetic variant in the IL-32 promoter affects IL-32γ expression and the production of proinflammatory cytokines led us to the hypothesis that this SNP could affect susceptibility to TC. A cohort of 139 TC patients and a control cohort of 138 healthy individuals were genotyped for the rs28372698 IL32 genetic variant. Different models of genetic association were tested, including a gene dose–dependent model (comparison of frequencies of all three genotypes), a dominant model (comparison of frequencies between homozygous wild-type [TT] + heterozygous [TA] versus homozygous mutant [AA]) and a recessive model (comparison of frequencies between homozygous wild-type [TT] versus heterozygous [TA] + homozygous mutant [AA]). Significant differences were observed in the distribution of ancient and derived genotypes in patients and controls when a dominant model was assumed (i.e. TT + TA versus AA). Furthermore, the comparison of frequencies of homozygous TT and heterozygous TA genotypes between the cohorts resulted in statistical significance. All these analyses point toward an increased frequency of the ancient T allele in the TC cohort, arguing for an increased risk to develop TC in the presence of the homozygous TT or the heterozygous TA genotype (Table II).

Association of IL-32 rs28372698 with TC clinical outcome
For the assessment whether the IL32 genetic variant influences clinical outcome of TC, clinical parameters were compared between TC patients with different IL32 genotypes (Table III). A significant association was observed between IL32 genotype and the cumulative RAI dose received by the patients, with the T allele overrepresented in the group of patients that received the highest RAI dose. In this respect, the low RAI dose of maximally 3.7 GBq was sufficient to
achieve remission for only 19% of patients homozygous or heterozygous for the T allele, whereas in the group of patients homozygous for the A allele, this dose was sufficient to achieve remission in 37% of the patients (P = 0.03). No statistically significant differences were observed for other clinical parameters (Table III).

**Discussion**

In this study, we demonstrate the association of a germ-line polymorphism in the promoter of the *IL32* gene with increased TC susceptibility and poor responsiveness of thyroid tumors to RAI treatment. Our studies into the functional consequences of this polymorphism revealed elevated expression of *IL32γ*, the most potent isoform of this cytokine, in individuals bearing the T allele and, as a consequence, increased production of cytokines in a model of LPS-stimulated human PBMCs. These data indicate that functional genetic variants in *IL32* influence the susceptibility to TC.

The inflammatory process plays a fundamental role in carcinogenesis. The immune system is on the one hand regarded as the central guardian for detection and subsequent eradication of malignant cells, but on the other hand, chronic or pro-tumorigenically skewed inflammation has been shown to have an important role in the induction and progression of the malignant process. Therefore, genetic variation in genes that affect the function of proteins involved in inflammation could influence susceptibility to survival and expansion of malignant cells, eventually leading to tumor formation and potentially metastasis. IL-32 is a central mediator of both inflammatory and oncogenic pathways as reflected by its role in autoimmune, infectious and malignant diseases (9,20,22,23).

In an initial set of experiments, we demonstrate mRNA and protein expression of IL-32, that is, the IL-32β and IL-32γ isoforms, in tissue specimens derived from either papillary or follicular TC tumors. These findings indicate that the IL-32β and IL-32γ isoforms, known as the most potent inflammatory modulators of all IL-32 isoforms (15,24), are present in TC tumor tissue and could therefore influence the inflammatory profile of the tumor microenvironment. Presence of IL-32 protein in TC tissue could not be confirmed by western blot (data not shown), indicating that protein levels might be too low to be detected by immunoblotting.

Stimulation experiments of PBMCs with the TLR4 ligand LPS were used as a model to investigate the functional consequences of the *IL32* genotypes on IL-32 mRNA expression and on the induced inflammatory profile. These studies revealed a 2-fold higher mRNA expression of IL-32 in cells isolated from individuals homozygous for the ancient T allele. Separate transcriptional analysis of the isoforms IL-32β and IL-32γ revealed that the increase in total IL-32 in these individuals is due to elevated IL-32γ expression. As reported previously, this might be due to differential methylation of the *IL32* promoter, which could affect the magnitude of gene expression (25). Furthermore, LPS-induced secretion of TNFα, IL-1β, IL-6 and IL-10 protein was elevated at least 2-fold in cells homozygous for the ancient T allele. These findings suggest that IL-32γ amplifies cytokine responses induced by TLR4 intracellular signaling. Interestingly, it has previously been demonstrated that TC cells express TLR4 and respond to stimuli that induce TLR4 signaling, suggesting that the observed effects of the *IL32* polymorphism in PBMCs could also apply to thyrocytes (26).

In additional studies, we assessed whether the rs28372698 *IL32* TA gene polymorphism was associated with different susceptibility to TC. Indeed, the ancient T allele of this SNP was significantly more prevalent in TC patients when compared with control individuals, supporting a role of IL-32 in the pathogenesis of TC. In addition to the association with TC susceptibility, the *IL32* polymorphism was also significantly correlated with the response to RAI treatment. In fact, the same ancient T allele that confers higher susceptibility to TC development also predisposes to a poor response of the tumor to RAI. After stratifying patients for their *IL32* genotype, it was observed that twice as many patients homozygous for the protective A allele were effectively treated with a low RAI dose as compared with patients heterozygous or homozygous for the T risk allele. These data indicate that increased levels of IL-32γ expression lead to decreased susceptibility to RAI treatment. Although the precise mechanism is not known, it is tempting to speculate that this effect is exerted through modulation of membrane expression of the sodium iodide symporter (NIS). IL-32γ could exert these effects on NIS expression either directly or indirectly through the modulation of cytokine responses. The cytokines TNFα and IL-1, of which production is influenced by the *IL32* polymorphism, are known to downregulate NIS expression (27,28). Inflammation induced loss of NIS expression indicates differentiation of TC tumor cells, leading to a more aggressive and therapy-resistant phenotype of the malignant cells (29–31). These mechanisms could provide an explanation for the increased TC susceptibility and the higher RAI unresponsiveness in individuals bearing the *IL32* rs28372698 ancient T allele.

An important point to be considered is that of the correction for multiple testing in this study. First, the assessment of only one polymorphism in relation to clinical parameters is one argument against the need for multiple testing. Second, one of the primary endpoints was the susceptibility to TC, which is by itself only one initial analysis that has been performed. Alternatively, correction for multiple testing could be applied to the secondary analysis of the association with RAI treatment that would lead to a loss of statistical significance. However, in addition with the genetic analysis, we also provide evidence of functional effects of the *IL32* polymorphism on the inflammatory response, and that is likely to represent the biological explanation for the observed genetic associations.

Several studies have demonstrated the proinflammatory effects of IL-32 in amplifying cytokine signaling, mechanism through which IL-32 aggravates immune-driven diseases (9,10,15,18). Furthermore, IL-32 expression in tumor tissue of lung cancer patients was shown

**Table II.** Difference in genotype frequencies between TC patients and controls, and the effect of the *IL32* genotypes on epithelial cell–derived TC susceptibility

<table>
<thead>
<tr>
<th>Gene dose–dependent model</th>
<th>Patients</th>
<th>Controls</th>
<th>Odds ratio (95% confidence interval)</th>
<th>P-value*</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>46 (33%)</td>
<td>64 (46%)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>72 (52%)</td>
<td>56 (41%)</td>
<td>1.79 (1.07–2.99)</td>
<td>0.027</td>
</tr>
<tr>
<td>TT</td>
<td>21 (15%)</td>
<td>18 (13%)</td>
<td>1.62 (0.78–3.39)</td>
<td>0.196</td>
</tr>
<tr>
<td>Total</td>
<td>139</td>
<td>138</td>
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<tr>
<td>Dominant model</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>46 (33%)</td>
<td>64 (46%)</td>
<td>1.75 (1.08–2.84)</td>
<td>0.024</td>
</tr>
<tr>
<td>TA + TT</td>
<td>93 (67%)</td>
<td>74 (54%)</td>
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<tr>
<td>Total</td>
<td>139</td>
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<td>Recessive model</td>
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<tr>
<td>AA + TA</td>
<td>118 (85%)</td>
<td>120 (87%)</td>
<td>1.19 (0.60–2.34)</td>
<td>0.622</td>
</tr>
<tr>
<td>TT</td>
<td>21 (15%)</td>
<td>18 (13%)</td>
<td></td>
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<tr>
<td>Total</td>
<td>139</td>
<td>138</td>
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</table>

*Logistic regression.
to be correlated with the presence of proinflammatory cytokines and, similar to our observations in TC, was associated with poor prognosis (14). On the contrary, others have reported a protective role of IL-32 in mouse models of melanoma and colon cancer (19). A potential explanation for these discrepancies is the differential role of IL-32 between cell types and its participation in different intracellular pathways. In fact, inhibition of cytokine mRNA decay has been described as a mechanism of IL-32-induced hyperinflammation, whereas the protective role of IL-32 on tumor cell proliferation is exerted by inhibition of the transcription factors nuclear factor-kB and signal transducer and activator of transcription 3 (15,19). Because the latter results were obtained by overexpression of endogenous human IL-32γ in murine cells, this might be the reason for these apparent conflicting results. Furthermore, it is known that supra-physiological concentrations of endogenous IL-32 lead to cell death. Previously, Arcaroli et al. (20) reported the genetic association of another IL32 polymorphism (the intronic rs12934561 SNP) with susceptibility and severity of infection-associated acute lung injury, indicating that genetic variation in IL32 influences the magnitude of inflammatory pathway induction. Nevertheless, this study is the first that describes functional consequences of a human IL32 polymorphism leading to increased inflammatory responses.

In conclusion, this study raises the concept of an important role for endogenous IL-32γ; the most potent IL-32 isoform, in modulation of the inflammatory response in humans that influences susceptibility to and progression of TC. In this respect, we show that an IL32 promoter polymorphism that increases IL-32γ expression is also associated with an increased susceptibility to TC and RAI unresponsiveness. In TC, this novel pathway may have promising therapeutic potential. However, several aspects of IL-32 biology and function in thyrocytes remain elusive. The molecular mechanisms mediated by IL-32 in the thyroid, including inflammatory pathways and the possible modulation of iodine handling molecules such as NIS, are to be elucidated. Furthermore, future studies should reveal whether the functional effects of the IL32 polymorphism observed in human white blood cells are also exerted in the TC tumor microenvironment.

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**References**

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