The common genetic variant rs944289 on chromosome 14q13.3 associates with risk of both malignant and benign thyroid tumors in the Japanese population

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Running title: Risk genotypes in FA and PTC

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Abstract

Background: Several single nucleotide polymorphisms (SNP) have been identified to be associated with the risk for differentiated thyroid cancer in populations of distinct ethnic background. The relationship of these genetic markers to a benign tumor of the thyroid, follicular adenoma (FA), is not well established.

Methods: In a multicenter retrospective case-control study, five thyroid cancer-related SNPs: rs966513 (9q22.33, *FOXE1*), rs944289 (14q13.3, *PTCSC3*), rs2439302 (8p12, *NRG1*), rs1867277 (9q22.23, *FOXE1*) and rs6983267 (8q24, *POU5F1B*) were genotyped in 959 cases of histologically verified FA, 535 papillary thyroid carcinomas (PTC), and 2766 population controls.

Results: A significant association was found between FA and rs944289 (p = 0.002; OR = 1.176, 95%CI 1.064–1.316), and suggestively with rs2439302 (p = 0.033; OR = 1.149, 95% CI 1.010 – 1.315). In PTC, significant associations were confirmed for rs965513 (p = 4.21E-04; OR = 1.587, 95%CI 1.235–2.000) and rs944289 (p = 0.003; OR = 1.234, 95%CI 1.075–1.408), newly found for rs2439302 (p = 0.003; OR = 1.266 95%CI 1.087–1.493) and rs1867277 (p = 1.17E-04; OR = 1.492, 95%CI 1.235–1.818), and was not replicated for rs6983267 (p = 0.082; OR = 1.136 95%CI 0.980–1.316) in this series. A significant correlation between rs2439302 genotype and relative expression of *NRG1* was detected in normal and tumor counterparts of PTC (about 10% decrease per each risk allele). *NRG1* expression also significantly correlated with that of *PTCSC3*.

Conclusions: Association of rs944289, which was previously known to confer risk for thyroid cancer, with FA, and the correlation between *PTCSC3* and *NRG1* expression demonstrates that predisposing genetic factors are partly common for benign and malignant thyroid tumors, and imply broader roles of the pathways they underlie in thyroid tumorigenesis, not limited to carcinogenesis.

Introduction

Follicular adenoma (FA), a benign thyroid neoplasm, together with two types of well-differentiated thyroid cancer, the follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC), are the most common tumors derived from thyroid follicular epithelium. The prevalence of PTC among thyroid malignancies is 85-90% and that of FTC about 10%, thus the ratio is approximately 9:1 (1). Variations in the prevalence may be observed between different geographic areas and ethnicities. In Japan, which is an iodine-sufficient region, the prevalence of PTC in 2003 was 92.5% and that of FTC was 4.8%, with a ratio of approximately 19:1 (2). The prevalence of clinically detectable FA is likely to be intermediate between PTC and FTC since in surgical specimens, the FA to FTC ratio is about 5:1 (3).

FA displays morphological similarities (as well as dissimilarities) with differentiated thyroid cancers displaying a follicular growth pattern, especially with FTC and the encapsulated follicular variant of PTC with minimal nuclear changes. Genetic alterations described in both FA and malignant thyroid tumors include mostly *RAS* mutations (4-7) and *PAX8/PPARG* rearrangements (8). The commonality of certain morphological and molecular features between FA and well-differentiated thyroid cancer prompts the question whether inherited genetic markers, namely single nucleotide polymorphisms (SNP) conferring risk for thyroid malignancy, may also associate with benign thyroid tumors.

Three genome-wide association studies performed in populations of European ancestry reported an association of rs966513 on chromosome 9q22.33 (upstream *FOXE1*, Forkhead box E1 (9,10)), rs944289 on 14q13.3 (*NKX2-1*, NK2 homeobox 1 neighborhood (9), later determined to regulate *PTCSC3*, Papillary thyroid carcinoma susceptibility candidate 3 (11)) and rs2439302 on 8p12 (*NRG1*, Neuregulin 1 (12)) with well-differentiated thyroid cancer. Independently, rs1867277 at 9q22.23 in *FOXE1* 5'UTR (13,14) as well as rs6983267

on 8q24 (upstream *POU5F1B*, POU class 5 homeobox 1B) conferring risk for several cancers were found to be associated with PTC using a target gene approach (15-17). Two SNPs, rs965513 and rs944289, have been replicated in the Japanese population (18). However, until recently it was unknown whether any of these SNPs were associated with FA. The only study performed so far claimed a significant association between rs944289 and "benign thyroid tumor" (FA and hyperplastic nodules combined) in the Han Chinese population (19). Of note, whether genetic and pathogenic mechanisms underlying FA (a tumor) and a hyperplastic nodule (thyroid hyperplasia) are identical is not well understood, and these two types of lesions are histopathologically distinct entities.

The primary goal of this work was to determine whether SNPs previously reported to confer risk for thyroid malignancy may also associate with FA. For this purpose we genotyped 959 Japanese patients with morphologically verified FA and 2766 population controls for the five SNPs. To make a comparison with thyroid cancer, we analyzed genotypes of 535 Japanese patients with PTC, expanding our previous study (18). In addition, to understand the relationship of rs944289 and rs2439302 to the regulation of *NKX2-1* and/or *PTCSC3*, and *NRG1* expression, respectively, we analyzed matched normal thyroid and tumor tissues from 81 patients with PTC.

Material and Methods

Patients and a control cohort

A total of 1162 formalin-fixed paraffin-embedded tissues from patients subjected to thyroid surgery between 1963 – 2011 for whom the diagnosis of FA was identified in the Nagasaki Tumor Registry files were collected from Nagasaki city hospitals and the National hospital organization Nagasaki Medical Center (Omura city); other participating centers were Kuma hospital (Kobe city) and Ishigaki clinic (Hamamatsu city).

Morphological diagnosis of FA was verified by experienced pathologists (A.B., M.N., M.I., T.H., T.B.) to exclude concomitant thyroid cancer and/or other types of thyroid nodules or nodular hyperplasia. Most slides (816/1162, 70.2%) were reviewed by at least two independent pathologists, and all slides were reexamined at least twice. After histological verification, 959 tissue specimens were confirmed as FA according to the WHO classification (20). A control population was sampled in Kyoto University (2766 blood specimens). Thirty-eight samples obtained from patients with PTC operated between 2010 – 2013 in Nagasaki University Hospital were added to our previous series of 507 samples (18). A summary of patients and controls is presented in Table 1.

The protocol of the study was approved by the ethical committees of all participating institutions.

DNA extraction

Genomic DNA was extracted from five 10 μm sections using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. All sections were taken from the blocks containing both FA tumor and normal thyroid tissue to ensure unbiased genotyping (21). DNA of sufficient quality and quantity (as measured with a Nanodrop ND-100 spectrophotometer) was obtained from all 959 FA samples. DNA samples that displayed poor PCR amplification and/or ambiguous base call signal on direct genotyping (presumably due to chemical modifications caused by extensive fixation in formalin, and long sample storage/age) were treated with borate-NaOH buffer (pH11.0) as described before (22). Genomic DNA from frozen PTC samples was extracted using the Proteinase K/Phenol-Chloroform method.

SNP genotyping

Genotyping was performed with pre-designed custom ABI TaqMan SNP assays (functionally tested primer/probe sets are listed in Supplementary Table 1) in accordance with manufacturer's guidelines on a Light Cycler 480 (Roche, Indianapolis, IN, USA) using TaqMan Genotyping Master mix (all reagents from Life Technologies, Foster City, CA, USA) and 10 ng genomic DNA per 10 µl reaction. Cycling conditions were as follows: denaturation at 95°C for 10 min followed by 50 cycles of [92°C for 15 sec and 62°C for 1 min] for all SNPs.

The control set included 2764 genotypes for rs965513 and 2766 for rs944289, which were extracted from the genome scans obtained previously with Illumina Human610-Quad BeadChip arrays (18). For rs2439302, rs1867277 and rs6983267, 2766, 2724 and 2759 genotypes, respectively, were imputed using genotypes of the International HapMap Project as reference.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from frozen tissues of normal and tumor counterparts of 81 PTC cases with Isogen (Nippon Gene, Toyama, Japan). 25 µg of isolated RNA was treated with RNase-free DNase I (Qiagen, Valencia, CA, USA) for 20 min at room temperature, and cleaned up and concentrated using a RNeasy Micro Kit (Qiagen, Valencia, CA, USA). 5 µg of DNA-free RNA was reverse-transcribed using MuLV Reverse Transcriptase, random hexamers and RNase Inhibitor (all reagents from Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 50 µl. Incubation was performed at 41ºC for 60 min followed by heat inactivation at 95ºC for 5 min.

An aliquot of 2 µl of cDNA was used in 25 µl PCR reactions containing 12.5 µl of 2xSybr Premix Ex Taq II (Takara, Otsu, Japan) and 400 pM of each primer for the target or

reference gene (*POLR2A*, polymerase (RNA) II (DNA directed) polypeptide A). All primers were designed using Primer Express 3.0 software (Applied Biosystems), Supplementary Table 2.

The quantitative real-time PCR (qRT-PCR) amplifications for each gene were performed in duplicates in a Thermal Cycler Dice Real Time System II (Takara, Otsu, Japan). All samples were run under the same PCR cycling conditions: 95ºC for 30 sec, then 40 cycles [95ºC for 5 sec and 59ºC for 30 sec] followed by dissociation curve analysis to ensure the signal from target amplicon. The average of the relative quantity of replicates was calculated by the $\Delta\Delta C_t$ method with built-in software.

Statistical analysis

Differences between case-control groups for each SNP was examined using logistic regression analysis in the multiplicative model of inheritance adjusted for age and sex. The associations for each SNP were evaluated in terms of *p*-value, odds ratio (OR) and its corresponding 95% confidence interval. Heterogeneity of the OR between FA and PTC was estimated with Breslow-Day test using the FREQ procedure with the CMH option in the SAS system (version 9.2; SAS Institute, Cary, NC, USA). Relationships between gene expression levels in the grouped samples were assessed with Wilcoxon signed-ranks or Kruskal-Wallis test as appropriate. Multivariate linear regression analysis was applied to examine a correlation between *NRG1* expression level and rs2439302 genotype, and *NKX2-1* and *PTCSC3* expression levels and rs944289 genotype.

Unless otherwise specified, all calculations were performed using SPSS 17.0 statistical software package (SPSS, Chicago, IL, USA). *p*-values less than 0.05 were regarded as indicating statistical significance in all statistical tests.

Results

Association analysis of FA and PTC

The number of samples that passed quality control, depending on the SNP, were 901 – 933 (of 959) in the FA series, 486 – 535 (of 545) of the PTC, and 2759 – 2766 (of 2766) in the population controls. Corresponding genotype call rates per SNP ranged between 0.940 – 0.973 in FA, 0.892 – 0.982 in PTC, and 0.997 – 1.0 in the controls (Supplementary Table 2).

In FA, two of five SNPs, rs944289 (p = 0.002; OR = 1.176, 95% CI 1.064 – 1.316) and rs2439302 (p = 0.033; OR = 1.149, 95% CI 1.010 – 1.315) displayed associations (Table 2). The latter, however, did not survive correction for multiple testing; therefore, its association with benign tumor should be considered suggestive.

In PTC, four SNPs were significant. Genotypes determined in the current study were pooled with those from the previous investigation (18) yielding association signals for rs965513 (p = 4.21E-04; OR = 1.587, 95% CI 1.325 – 2.000) and rs944289 (p = 0.003; OR = 1.234, 95% CI 1.075 – 1.408) (Table 2). The significance of associations for both SNPs became stronger as compared to our earlier report, likely due to the larger sample size (6.59E-5 *vs*. 1.27E-4 for rs965513, and 8.82E-4 *vs*. 1.21E-2 for rs944289 if trend exact test is applied as in ref. 18). The newly assessed rs2439302 (p = 0.003; OR = 1.266, 95% CI 1.087 – 1.493) and rs1867277 (p = 1.17E-04; OR = 1.492, 95% CI 1.235 – 1.818) were also significant; this is the first report in the Japanese population. In contrast, association of rs6982367 could not be confirmed (p = 0.082; OR = 1.136, 95% CI 0.980 – 1.316).

Comparison of a SNPs' effect sizes in FA and PTC demonstrated that rs944289 and, likely, rs2439302 associated with both FA and PTC (p_{het} = 0.646 and 0.324, respectively), while rs965513 and rs1867277 in the *FOXE1* region associated with PTC (p_{het} < 0.03) (Table 2).

Correlation between rs944289 genotype and relative expression of *NKX2-1* and *PTCSC3*

rs944289 localizes 336 kb centromeric and downstream to *NKX2-1*, which after the discovery of an association of rs944289 with thyroid cancer has been initially proposed as a gene which this SNP may tag (9); the link between *NKX2-1* and rs944289 has never been explored before. Later on, rs944289 was shown to correlate with *PTCSC3* expression localized 3.2 kb downstream of this SNP (11). We examined the relationship of rs944289 with expression of the two genes in the available tissues from patients with PTC.

NKX2-1 expression levels correlated significantly between paired normal and tumor counterparts (Spearman's r = 0.419, p = 1.513E-4), but did not differ significantly between them (p = 0.509; Fig. 1A). *PTCSC3* expression also correlated between normal and tumor tissue (Spearman's r = 0.331, p = 0.003), yet in contrast to *NKX2-1*, it was significantly lower in the latter (p = 3.494E-5; Fig. 1C).

NKX2-1 expression did not associate with the rs944289 genotype in subgroup analysis (Fig. 1B) and in the regression model (Table 3). For *PTCSC3*, a statistically significant decrease in the expression level in tumor tissue was found only in heterozygous patients ($p < 0.01$), likely due to the larger sample size of this subgroup (hence, statistical power), although lower median levels were consistently observed for all genotypes (Fig. 1D). In the regression model, *PTCSC3* expression did not correlate significantly with the rs944289 genotype (Table 3).

Correlation between rs2439302 genotype and relative expression of *NRG1* in PTC

NRG1 expression levels weakly but significantly correlated between the paired tissue specimens (Spearman's r = 0.308, p = 0.005), and the level was significantly higher in normal thyroid as compared to PTC tumor tissue (p = 0.037; Fig. 2A).

rs2439302 is located in intron 1 of *NRG1*, and may be expected to influence its

expression. Indeed, homozygous carriers of the rs2439302 risk allele G displayed a significantly lower *NRG1* level in both normal thyroid and tumor tissues as compared to patients homozygous for the common allele C ($p < 0.05$, Fig. 2B). Furthermore, multivariate linear regression analysis detected a significant correlation between *NRG1* expression and rs2439302 genotype indicating about 10% decrease in the relative *NRG1* expression per each risk allele in both tumor and normal thyroid tissues (Table 3).

Relationship between relative expression of *PTCSC3* and *NRG1*, and combination effect of rs944289 and rs2439302 variants

Ectopic overexpression of *PTCSC3* in thyroid cancer cell lines has been reported to affect several canonical pathways, and neuregulin signaling was the third among top five (p = 9.54E-4) (11). In our PTC series, a concordant decrease of *PTCSC3* and *NRG1* levels were observed in tumor tissue as compared to normal thyroid. We therefore examined the correlation between *PTCSC3* and *NRG1* expression and found that in both types of tissues the levels were directly proportional and correlated highly significantly (Spearman's r = 0.692, p = 8.266E-13 for normal thyroid, and r = 0.336, p = 0.002 for PTC tumor tissue; Supplementary Fig.1).

We further assessed whether there is an interaction between rs944289 and rs2439302. No evidence of an epistatic effect was obtained in regression analysis (p = 0.521 for FA and p = 0.343 for PTC). However, there were additive effects of the two SNPs, suggesting that carriers of two or more of risk alleles are at greater risk for both FA and PTC than individuals with one risk allele (Fig.3). The additive effect of rs944289 and rs2439302 was also supported by a consistent increase in effect size observed in the subgroups with increasing number of risk alleles (Supplementary Table 3).

Discussion

Rapid progress in the identification of genetic markers of susceptibility to thyroid cancer prompted us to investigate their association with not only thyroid malignancy but also with a benign thyroid tumor, and to examine the relationship between rs944289 and rs2439302 with expression levels of the genes they may point at: *NKX2-1* and/or *PTCSC3*, and *NRG1*, respectively.

We found that rs944289 was associated with both FA and PTC. This SNP is localized in the intergenic region of chromosome 14q13.3, and was initially proposed to possibly tag the thyroid-associated *NKX2-1* gene (9). *NKX2-1* encodes thyroid transcription factor 1 (TTF1), a key regulator of thyroid, lung and brain morphogenesis (23). A relationship between reduced expression of Nkx2-1 and thyroid tumors was established in *Nkx2-1* thyroid-conditional-hypomorphic mice treated with a nitrosamine-based carcinogen followed by sulfadimethoxine as a promoter, in which an increased incidence of FA was observed (24). In humans, our analysis showed no evidence of a correlation between rs944289 and *NKX2-1*, and of differential expression of the gene in normal and PTC tissues, indicating that this SNP is unlikely to point at *NKX2-1* as at a risk factor for thyroid tumorigenesis.

In an independent functional study, rs944289 was shown to regulate expression of *PTCSC3*, a lincRNA gene with tumor suppressor properties in thyroid cancer cell lines (11). *PTCSC3* expression was significantly downregulated in PTC as compared to normal thyroid in our series, in line with the mentioned study. Restoration of *PTCSC3* expression in cell lines inhibited cell growth and affected the expression of genes corresponding to three networks: i) DNA replication, recombination and repair, gene expression, amino acid metabolism, ii) cellular movement, tumor morphology, cell death, and iii) cellular assembly and organization, cellular function and tissue morphology (11). Of note, all these ontological categories except for cell motility are well applicable to both malignant and benign tumors.

The risk allele of rs944289 [T] was shown to disrupt the C/EBPα and C/EBPβ transcription factors' binding site in the *PTCSC3* promoter, and a significant difference in *PTCSC3* expression was found between [TT] homozygotes and heterozygous [CT] patients (11). In our work, we could not detect a correlation between rs944289 genotype and *PTCSC3* expression. A possible explanation could be that rs944289 is not the only regulatory element of *PTCSC3*; there may be another SNP or a polymorphism of a different type in linkage disequilibrium with rs944289 that affects *PTCSC3* expression.

A significant association of rs2439302 located in the *NRG1* intron and risk for PTC and, likely, with FA was a novel finding in the Japanese population. After the initial report in thyroid cancers (12), replication studies have been performed in patients with PTC from Poland and the USA (25), and in a Chinese cohort (19). All studies were concordant in confirming an association of rs2439302 with PTC. However, no signs of association were found with benign thyroid nodules in the Chinese study ($p = 0.7779$) (19). The reasons for the discrepancy between the latter and our work could be that rs2439302 may associate with FA but not with hyperplastic nodule, or it may stem from ethnic differences in the genetic predisposition between the two populations.

The relative expression of *NRG1* was found to be decreased by approximately 10% per each allele [G] of rs2439302 in both normal and tumor counterparts of PTC. *NRG1* expression was overall lower in tumor tissue and tended to be lower in tumor tissue as compared to normal thyroid for all rs2439302 genotypes. Mechanistically, this tendency could be explained by the fact that rs2439302 localizes within the CTCF (CCCTC-binding factor, a transcription factor) binding region as observed in several cell lines of non-thyroid origin (19). CTCF expression is decreased in thyroid cancer tissues, which may cause corresponding downregulation of *NRG1*. Alternatively, our *in silico* analysis suggested that *NRG1* suppression in PTC may be the result of RXR/VDR (Retinoid X receptor/Vitamin D

receptor) biding to negative vitamin D response elements (nVDREs) modulated by both rs2439302 and excessive *RXRG* and *VDR* levels in tumor tissues (Supplementary Fig. 2). However, additional experiments are needed to test this hypothesis.

Interestingly enough, the *PTSC3* and NRG1 pathways appear to be interrelated, at least in PTC (FA tissues of appropriate quality were not available for the study). In agreement with the previous work (11), we observed a significant positive correlation between the two genes. The mechanism by which *PTCSC3* modulates *NRG1* expression remains unknown. NRG1 is a HER3 ligand; it can activate proliferative and survival MAPK and AKT signaling pathways under conditions causing HER2/HER3 dimer induction in thyroid cancer cells (26). The definite association of *PTCSC3* and suggestive association of *NRG1* with FA obtained in our investigation imply that the pathways affected by *PTCSC3* derangement are functioning not only in cancer but may also play certain role in the development of benign thyroid tumors.

In contrast to rs944289 and rs2439302 (*PTCSC3* and *NRG1*, respectively), which associated with both PTC and FA, the two SNPs in the *FOXE1* locus on chromosome 9q22.33, rs965513 and rs1867277, associated with a risk for PTC. The relationship of FOXE1 to thyroid cancer may additionally be illustrated by immunohistochemical studies of FOXE1 expression in thyroid tumors (27-29). There has been a profound difference between staining patterns in different types of neoplastic tissues. Cytoplasmic FOXE1 staining was the strongest in medullary thyroid carcinoma, followed by PTC, then FTC and the weakest in FA. Thus, cytoplasmic translocation of FOXE1 appears to be a phenotypic hallmark of thyroid cancer.

An association between rs6983267 located on chromosome 8q24 (upstream of *POU5FB1*) and FA was not detected. We also could not replicate an association with PTC in the Japanese population, yet the effect direction (OR = 1.136) was the same as in individuals of European ancestry (15, 17), and an association signal of borderline significance (p =

0.082) was noted. A plausible reason for the negative finding could be insufficient statistical power achievable with the available PTC series. Our results, therefore, do not rule out the possibility of an association of rs6983267 with thyroid cancer in the Japanese population and need to be reexamined in a larger sample. It is also worth noting that despite no significant associations with FA were detected for rs965513 and rs1867277 (*FOXE1*), and rs6983267 (*POU5FB1*), their effects (in terms of ORs) were generally consistent between FA and PTC (Table 2), suggesting that these SNPs might also affect predisposition to FA to some extent, but their contribution is weaker compared to that seen in thyroid cancer.

In conclusion, our study demonstrates that a common genetic variant, rs944289 (*PTCSC3*), previously reported to be associated with thyroid cancer, also associates with FA. rs2439302 (*NRG1*) displayed a suggestive association with FA and its expression level correlated with that of *PTCSC3*. This indicates that the mechanisms mediated by *PTCSC3* and NRG1 are likely to play roles not only in carcinogenesis but more broadly in thyroid tumorigenesis. SNPs in the *FOXE1* locus (rs965513, and rs1867277, the latter was ~~which is~~ newly genotyped in the Japanese population) are definitely associated with the risk for thyroid cancer.

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Author Disclosure Statement

No competing financial interests exist.

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650 Table 1. Demographic characteristics of study participants

	Total	Males	Females	% Females	Age range, y.o.	Age, mean ± SD
FA	959	132	827	86.2	9 – 87 [a]	48.2 ± 16.0
PTC	545	84	461	84.6	13 – 87 [a]	51.2 ± 15.8
Controls	2766	1019	1747	63.2	20 – 79 [b]	50.1 ± 15.4

651

652 [a] Age at diagnosis

653 [b] Age at sampling

Table 2. Association analysis for SNP markers in the Japanese FA and PTC series

SNP	Allele [a]		Chr	Nearest gene	Frequency of variant allele			Association FA		Association PTC		p_{het} [d]
	Ref	Var			FA	PTC	Ctrl	OR (95% CI) [b]	*p*	OR (95% CI) [b]	*p*	
rs965513 Sample	A*	G	9q22.33	*FOXE1*	0.064 931	0.090 517	0.057 2671	1.111 (0.885–1.389)	0.370	1.587 (1.235–2.000)	4.21E-04	0.026
rs944289 Sample	C	T*	14q13.3	*PTCSC3*	0.455 918	0.465 505	0.409 2682	1.176 (1.064–1.316)	0.002	1.234 (1.075–1.408)	0.003	0.646
rs2439302 Sample	G*	C	8p12	*NRG1*	0.224 933	0.242 535	0.195 2724	1.149 (1.010–1.315)	0.033 [c]	1.266 (1.087–1.493)	0.003	0.324
rs1867277 Sample	A*	G	9q22.33	*FOXE1*	0.116 936	0.155 513	0.110 2724	1.086 (0.917–1.276)	0.351	1.492 (1.235–1.818)	1.17E-04	0.010
rs6983267 Sample	G*	T	8q24	*POU5FB1*	0.341 901	0.368 486	0.341 2759	1.010 (0.901–1.124)	0.880	1.136 (0.980–1.316)	0.082	0.191

[a] The reference (Ref) and variant (Var) alleles according to NCBI Build 37.5; the risk allele is indicated with an asterisk

[b] Odds ratio (OR) was calculated for the risk allele with a confidence interval (CI) of 95%

[c] $p = 0.082$ after correction for multiple testing (FDR)

[d] Heterogeneity of the OR between FA and PTC, the Breslow-Day test

661 Table 3. Relationship between genotype and gene expression level [a]

SNP	Gene	Normal thyroid			Tumor tissue		
		B [b]	95% CI [c]	*p*	B [b]	95% CI [c]	*p*
rs944289	*NKX2-1*	0.092	-0.180 – 0.364	0.503	0.291	-0.015 – 0.596	0.062
	PTCSC3	-0.267	-0.873 – 0.339	0.382	0.308	-0.095 – 0.711	0.133
rs2439302	*NRG1*	-0.109	-0.179 – 0.039	0.003	-0.096	-0.157 – 0.036	0.002

662

663 [a] Multivariate linear regression analysis, the additive model adjusted for age and sex

664 [b] B-coefficient in the model

665 [c] 95% confidence interval for B

Figure legends

Figure 1. Relative *NKX2-1* and *PTCSC3* expression in normal thyroid (open boxes) and PTC tumor (hatched boxes) tissues. (A and C) Comparison of 81 paired tissue samples; asterisk indicates significant difference (p = 3.494E-5 by Wilcoxon matched-pairs signed-ranks test). (B and D) Relative expression by rs944289 genotype; double asterisk indicates significant difference ($p < 0.01$ by Kruskal-Wallis test). The bottom and top lines of the box represent the values corresponding to the 25th and the 75th percentiles, respectively; the line inside the box represents the median; the range shown by the whiskers corresponds to the 1^{tst} and the 99th percentiles.

Figure 2. Relative *NRG1* expression in normal thyroid (open boxes) and PTC tumor (hatched boxes) tissues. (A) Comparison of 81 paired tissue samples; asterisk indicates significant difference (p = 0.037 by Wilcoxon matched-pairs signed-ranks test). (B) Relative *NRG1* expression by rs2439302 genotype; double asterisk indicates significant difference ($p < 0.05$ by Kruskal-Wallis test).

Figure 3. Joint effect of risk alleles of rs944289 (*PTCSC3*) and rs2439302 (*NRG1*). Distributions of risk alleles were analyzed with the Chi-square test in patients with FA or PTC and in the control individuals with known genotypes for both SNPs. The differences in allele counts between patients and controls in the subgroups with two or more risk alleles are shown in comparison with corresponding subgroups with zero risk alleles.

Supplementary Figure 1. Correlation between relative *PTCSC3* and *NRG1* expression in normal thyroid and PTC tumor tissues. Solid lines are linear best-fit; departure from linearity was insignificant, $p > 0.2$ in both analyses. Normal thyroid tissue: slope = 0.086 ± 0.012 (standard error), $p = 1.568E-10$. PTC tumor tissue: slope = 0.085 ± 0.017, $p = 2.692E-6$.

Supplementary Figure 2. DNA sequence around rs2439302. RXR/VDR (Retinoid X receptor/Vitamin D receptor) binding site is shown in bold. [S] denotes the rs2439302 G/C polymorphism. Boxed are putative negative vitamin D response elements (nVDREs).

Using the MAPPER database (s1), rs2439302 was found to reside in a suboptimal but identifiable RXR/VDR binding site, and that nonrisk allele [C] disrupts it. RXR/VDR heterodimers are transcription factors occurring in multisubunit complexes that can stimulate or repress gene expression involved in diverse biological processes. A bioinformatic search of three pooled sets of expression microarray data (s2-s4) in the Oncomine database (https://www.oncomine.org/) revealed that the expression of *RXRG* (Retinoid X receptor gamma) is 1.7-8.6-fold (p = 6.92E-9) and *VDR* is 1.2-1.9-fold (p = 0.003) increased in PTC as compared to normal thyroid. *RXRG* upregulation in PTC and thyroid cancer cell lines has been also reported in other works (s5,s6). Several putative nVDREs in the rs2439302 area could be identified, one of which overlaps with the SNP. Enhanced binding of RXRG/VDR to it might explain both genotype-dependent and overall downregulation of *NRG1* expression in PTC.

References to Supplementary Fig. 2

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Supplementary Table 1. Number of successfully genotyped samples and corresponding call rates in the groups of Japanese patients with follicular adenoma, papillary thyroid carcinoma, and in population controls

SNP	TaqMan primer/probe set	FA [a] N=959 [b]	PTC [a] N=545 [b]	Controls N=2766 [b]
rs965513	C_1593670_20	931 (0.971) [c]	517 (0.949)	2764[d] (0.999)
rs944289	C_1444137_10	918 (0.957)	505 (0.927)	2766[d] (1.0)
rs2439302	C_16238367_10	933 (0.973)	535 (0.982)	2766[e] (1.0)
rs1867277	C_11736668_10	936 (0.959)	513 (0.941)	2724[e] (0.985)
rs6983267	C_29086771_20	901 (0.940)	486 (0.892)	2759[e] (0.997)

[a] Genotyped using TaqMan assay

[b] The maximal number of samples in the group

[c] Here and throughout the table: the number of samples with genotype calls and (call rate)

[d] Extracted from in-house genome scan database

[e] Imputed using genotypes of International HapMap Project as reference

771 Supplementary Table 2. Primers for real-time quantitative PCR analysis and amplicon size

772

Gene	Gene ID	Primer direction	Primer sequence	Amplicon size, bp
NRG1	3084	Forward Reverse	5’-GATTCAAGTGGTTCAAGAATGGGAA-3’ 5’-CATATACTCTCCAGAATCAGCCAGTGA-3’	134
NKX2-1	7080	Forward Reverse	5’-CATGAGGAACAGCGCCTCT-3’ 5’-CCCATGCCGCTCATGTT-3’	114
PTCSC3	100886964	Forward Reverse	5’-AGCTCCCAGGGAGATTAATGCA-3’ 5’-GGGAGGCCAAGGTGGGAA-3’	99
POLR2A	5430	Forward Reverse	5’-GCCCGCTGCGCACCATCAAG-3’ 5’-GGCGGCCTCCCTCAGTCGTC-3	117

773

774 Supplementary Table 3. Effect size in case-control subgroups with different numbers of risk-associated alleles of

775 rs944289 (*PTCSC3*) and/or rs2439302 (*NRG1*) in FA and PTC

776

Number of risk alleles	Controls	FA			PTC		
		Patients	OR (95% CI)	*p* [a]	Patients	OR (95% CI)	*p* [a]
0	599	180	1.000	Reference	84	1.000	Reference
1	1093	331	0.992 (0.807-1.221)	0.984	196	0.782 (0.595-1.029)	0.091
2	754	305	1.346 (1.088-1.666)	0.007	157	1.485 (1.116-1.976)	0.008
≥3	196	92	1.562 (1.159-2.106)	0.004	65	2.365 (1.647-3.396)	<1E-4

777

778 [a] Calculated with the Chi-square test for the allele counts in subgroups of patients and controls with one or more risk alleles as compared to

779 corresponding subgroups with zero risk alleles

780

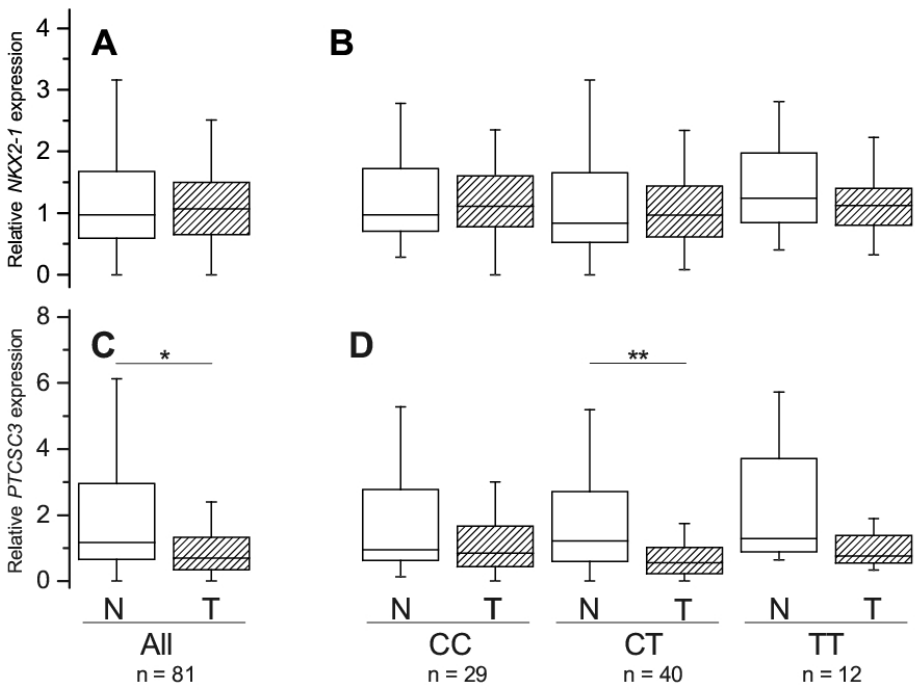
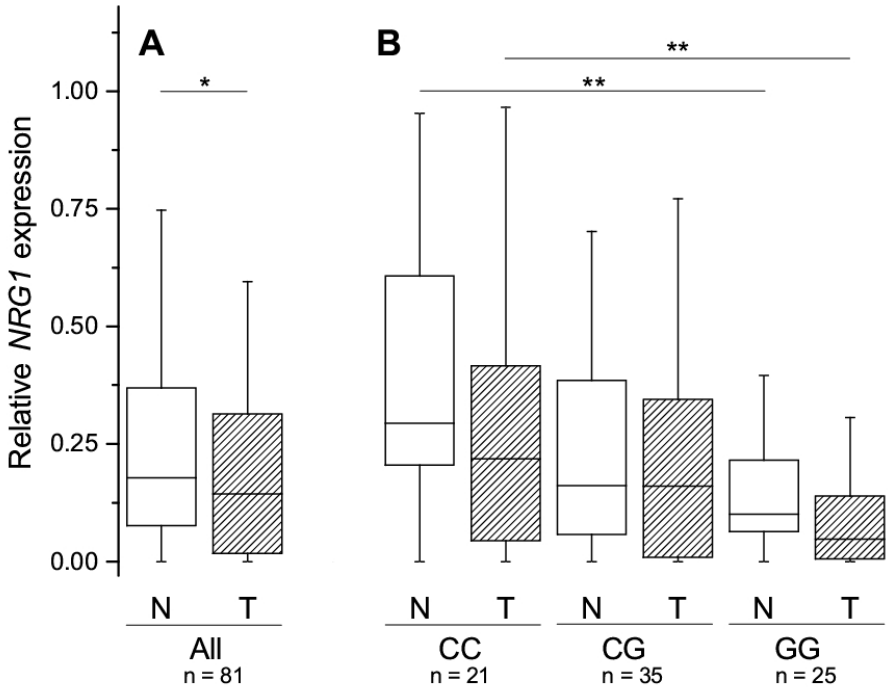

A
B
C
D
Relative NKX2-1 expression
Relative PTCSC3 expression
0
1
2
3
4
0
2
4
6
8
*
**
N
T
All
n = 81
CC
n = 29
CT
n = 40
TT
n = 12

Fig. 1


A
B
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Relative NRG1 expression
1.00
0.75
0.50
0.25
0.00
N
T
All
n = 81
CC
n = 21
CG
n = 35
GG
n = 25

Fig. 2

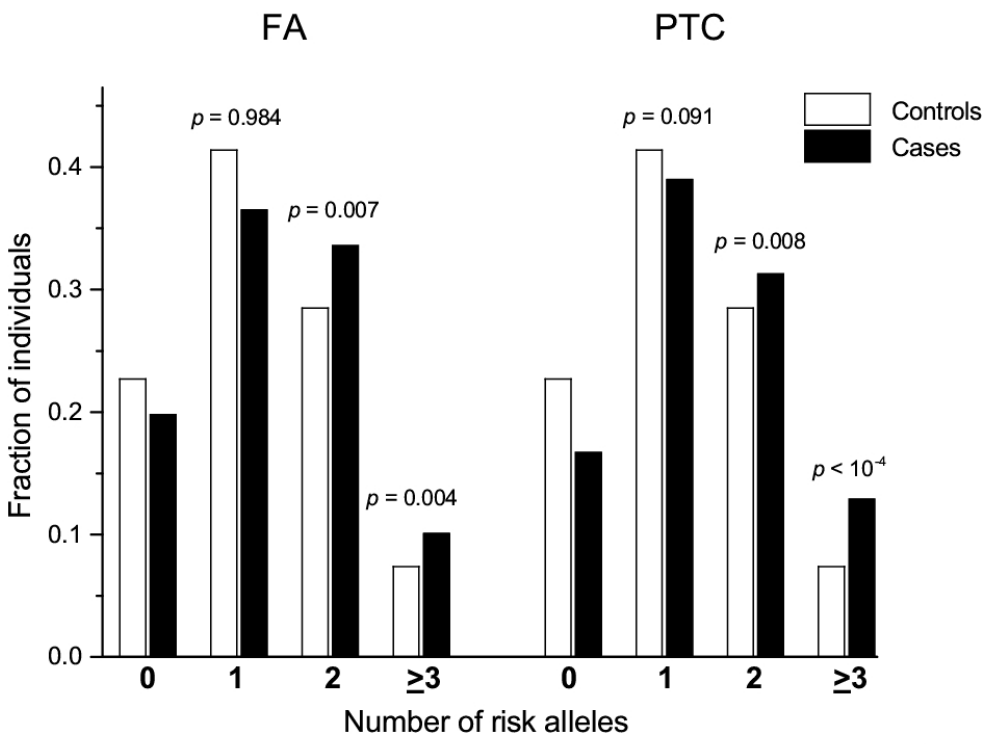
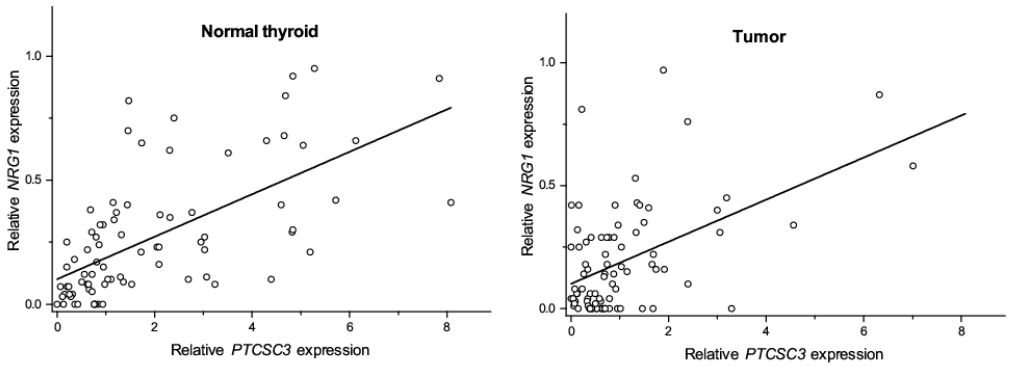
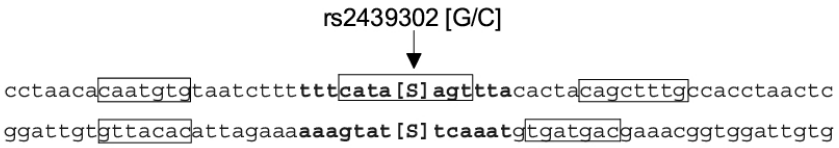

FA
PTC
Controls
Cases
p = 0.984
p = 0.007
p = 0.004
p = 0.091
p = 0.008
p < 10-4
Fraction of individuals
0.0
0.1
0.2
0.3
0.4
0
1
2
≥3
0
1
2
≥3
Number of risk alleles

Fig. 3



Supplementary Fig. 1



Supplementary Fig. 2