Papillary Thyroid Carcinoma: Association Between Germline DNA Variant Markers and Clinical Parameters

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Background: Papillary thyroid cancer (PTC) is reported to be highly heritable in epidemiological studies. Genome-wide association studies (GWAS) have uncovered several variants associated with PTC predisposition. It remains unknown whether these variants might contribute to better clinical stratification of PTC patients.

Methods: In order to assess the usefulness of germline genetic analyses in the management of PTC patients, the genotypes of five variants (rs965513, rs944289, rs116909374, rs2439302, and rs966423) were determined in 1216 PTC patients and 1416 controls. Additionally, the expression of seven genes located close to GWAS variants (*PTCSC3*, *MBIP*, *NKX2-1*, *FOXE1*, *DIRC3*, *PTCSC2*, and *NRG1*) were measured in 73 PTC paired tumor/normal tissues, respectively. Next, the association was analyzed between the genotypes of the germline variants and the levels of gene expression with clinical/pathological features such as age, sex, TNM staging, multifocality status, extrathyroidal expansion, and MACIS score.

Results: The risk allele of rs965513 was associated with larger tumor size ($p=0.025$) and extrathyroidal expansion (odds ratio [OR] = 1.29, $p=0.045$). The variant rs2439302 showed association with lymph node metastasis (OR = 1.24, $p=0.016$), and multifocality status of the tumor (OR = 1.24, $p=0.012$). The expression of *MBIP* was associated with T stage ($p=0.010$). *MBIP* and *PTCSC3* displayed lower expression in PTC tissue in males than in females ($p=0.025$ and $p=0.036$, respectively). *NKX2-1* displayed lower expression in patients with N1 stage ($p=0.040$).

Conclusions: The studied germline risk alleles predisposing to PTC were associated with a more aggressive course of the disease reflected by larger tumor diameter, higher multifocality rate, and more advanced N stage at the time of diagnosis. These results show that germline variants not only predispose to PTC but also might impact its clinical course. However, these associations were only moderate, and further large multi-ethnic studies are required to evaluate the usefulness of these germline variants in the clinical stratification of PTC patients.

Introduction

EVIDENCE FOR A GENETIC BASIS of papillary thyroid carcinoma (PTC) is one of the strongest among all cancers, as demonstrated by case-control studies (1,2). Despite the solid evidence of heritability, only a handful of variants have been convincingly implicated in PTC pathogenesis so far. The paucity of Mendelian gene mutations found in the etiology of PTC has suggested that the genetic etiology of PTC is primarily multifactorial (3,4). The advent of genome-wide association studies (GWAS) constituted a breakthrough in the ability to study the genetics of complex diseases. Single nucleotide polymorphisms (SNPs) associated with a low to medium risk for PTC have been described in several GWAS studies (5–9). The first variants uncovered by GWAS were

rs965513 (9q22), rs944289 (14q13), rs116909374 (14q13), rs2439302 (8p12), and rs966423 (2q35) (5,6). Characterizing the mechanisms by which these variants increase PTC risk has been difficult, just as in many other cancers. Major progress has occurred in the study of rs944289 and rs965513. Located in the promoter of a long non-coding RNA (lncRNA) gene named *Papillary Thyroid Carcinoma Susceptibility Candidate 3* (*PTCSC3*), the risk allele of rs944289 impacts the expression of *PTCSC3* by altering the binding affinity for the transcription factors C/EBP α and C/EBP β (10). *PTCSC3* was shown to possess tumor suppressor properties by inhibiting the motility and invasion potential of PTC cells through the suppression of *S100A4* transcription (11). The variant rs965513 at 9q22 is located in close vicinity of the *Forkhead Box E1* (*FOXE1*) gene. Recent studies have

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uncovered a lncRNA gene (*Papillary Thyroid Carcinoma Susceptibility Candidate 2* [*PTCSC2*]) overlapping with rs965513 (12). It was shown that *PTCSC2* was downregulated in PTC tissue compared with adjacent unaffected thyroid tissue, and that the rs965513 risk allele was associated with lower expression of *PTCSC2* in unaffected thyroid tissue. Ectopic expression of *PTCSC2* in a PTC cell line (BCPAP) impacted genes involved in cell cycle and cancer (12). Rs965513 is part of a haplotype block that regulates *FOXE1* and *PTCSC2* expression through long-range enhancer elements (13). A role for *FOXE1* in PTC development has been proposed in several studies (14–17).

Many studies have shown that somatic events in PTC tissue such as *BRAF* and *RAS* mutations are associated with clinical outcomes and may perhaps be important for the planning of surgery and post-surgery management (18–21). The best characterized marker of PTC aggressiveness is the somatic *BRAF*^{V600E} mutation, which occurs in approximately 40% of all PTC cases (22). *BRAF*^{V600E} has been associated with extrathyroidal invasion and lymph node metastases (20,23). In a large multicenter study, the *BRAF*^{V600E} mutation was associated with PTC recurrence and was independent of other conventional clinicopathologic risk factors (18). Other studies suggest that the combination of *BRAF*^{V600E} with mutations in the *TERT* promoter may better identify a subgroup of PTCs with more aggressive behavior (24). However, the clinical utility of these analyses is limited because somatic molecular testing is mostly performed postoperatively, since tumor tissue for DNA analysis is necessary. Notably, novel assays such as ThyroSeq (CBLPath, Rye Brook, NY) allow tumor DNA to be tested from material obtained by fine-needle aspiration. These tests provide good sensitivity and specificity for characterizing cytology samples with atypia of undetermined significance/follicular lesion of undetermined significance (25). The lack of data assessing the usefulness of germline variants in the clinical stratification of PTC patients prompted us this investigation of the potential association of germline SNPs and the expression of their potential target genes with clinical features.

Materials and Methods

Subjects and data collection

All studies were approved by the Institutional Review Board at the Ohio State University (OSU). DNA from blood was analyzed for 1216 patients with PTC. These comprised patients with classic PTC (cPTC; *n* = 891), follicular variant PTC (fvPTC; *n* = 243), and other less common variants (*n* = 82). Of this cohort, 620 cases and 805 controls were used in a previous study (26). Individuals were recruited from a multidisciplinary thyroid tumor clinic at the OSU Wexner Medical Center. In addition to blood samples, 73 paired tissue samples (unaffected thyroid and tumor tissue) were collected from individuals with PTC. Clinical and histopathological data of all the cases were extracted from the patients' charts and electronic medical records. The MACIS score—a prognosis score defined by using metastasis, age, completeness of resection, invasion, and tumor size—was analyzed (27). It was not possible to assemble data about the completeness of tumor resection, and the assumption was made for MACIS score calculation that all the samples were completely resected.

DNA samples from a group of individuals from Ohio (*n* = 1416) with no personal and/or family history of thyroid

disease were provided by the OSU Medical Center Human Genetics Sample Bank. All the subjects gave written informed consent before participation. They completed a questionnaire that included demographic, medical, and family history information.

DNA extraction and genotyping

Genomic DNA was extracted from blood according to standard phenol-chloroform extraction procedures and stored at 4°C. Normal and PTC tissue samples were snap frozen in liquid nitrogen and stored at –80°C. Total RNA from thyroid tissue was extracted using Trizol solution according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Genotyping

DNA from the cases and controls was whole-genome amplified using standard protocols based on the Phi29 polymerase (Thermo Fisher Scientific, Waltham, MA). Genotyping of all five SNPs was performed by applying the Nanogen Centaurus single-track assay (28). The whole-genome DNA amplification and genotyping was performed by deCODE Genetics (Reykjavik, Iceland).

Quantitative PCR

Total RNA (1 µg) was first treated with DNase-1 (Ambion, Waltham, MA) and then reverse transcribed to cDNA with the High Capacity Reverse Transcriptase kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Fast TaqMan gene expression assays (Life Technologies, Carlsbad, CA) were applied for measuring *NKX2-1*, *FOXE1*, *MBIP*, *DIRC3*, and *NRG1* expression. For the assessment of *PTCSC2* and *PTCSC3* expression, a custom-made TaqMan assay was used (Life Technologies).

The quantitative polymerase chain reactions were performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The conditions for the Fast TaqMan assays were as follows: 95°C for 5 sec followed by 40 cycles at 95°C, 5 sec of denaturation, and 60°C, 5 sec of annealing/elongation. For the custom-made TaqMan assay, the conditions were as follows: 95°C for 10 min followed by 40 cycles of denaturation (95°C, 15 sec) and annealing/elongation (60°C, 1 min). The *GAPDH* gene (Fast TaqMan gene expression assay) was used as an internal control, and the formula $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{(GENE)} - Ct_{(GAPDH)}$, was employed to calculate the relative transcript levels for each analyzed gene.

Cumulative genetic risk score

Each SNP was scored 0, 1, or 2 in order to indicate the number of PTC risk alleles of a single variant in a given case. Two types of cumulative genetic risk scores (CGRS) were calculated: unweighted CGRS by simply adding risk alleles, and weighted scores (wCGRS) by adding the weighted disease allele counts by logarithm odds ratio, log(OR), of each SNP (26). Samples with any missing genotypes were not included in the genetic risk score calculations.

Statistical analysis

Generalized linear models (GLM) were applied to assess the association between phenotypes/clinical features and

genetic variation with allelic model or cumulative risk scores. Both univariate analysis and multivariate analysis adjusting for age, sex, race/ethnicity, and familiarity were performed. In the smaller size groups of fvPTC and microcarcinomas (microPTC), the analysis was adjusted only for age and sex. Association analysis between gene expression and categorical clinical factors were performed by applying non-parametric Wilcoxon tests or Kruskal–Wallis tests, while continuous clinical variables were evaluated by applying Spearman rank correlation tests. All the reported *p*-values are two-sided, and a *p*-value of <0.05 was considered to be significant. The R statistical program (www.r-project.org/) was used to perform all the analyses.

Results

Patient characteristics

Table 1 summarizes the characteristics of the patients. A standard TNM staging system was used to characterize the cases (29). Most of the cases were of cPTC histology (*n* = 891; 73.3%). The second largest histology subgroup was fvPTC (*n* = 243;

20.0%). The rest of the cases were as follows: tall-cell variant (*n* = 22; 1.8%), oncocytic variant (*n* = 17; 1.4%), columnar-cell variant (*n* = 5; 0.4%), diffuse sclerosing variant (*n* = 2; 0.2%), clear-cell variant (*n* = 1; 0.1%), cystic type (*n* = 1; 0.1%), and not otherwise specified PTC variant (*n* = 34; 2.8%). Based on the World Health Organization definition, 328 (27.0%) cases were classified as microPTC (tumor in the longest diameter ≤1 cm). The female:male ratio was 3:1. The vast majority of the subjects were Caucasian (*n* = 1146; 94.2%). More than 50% of the tumors were limited to the thyroid (T1), while 38.4% of cases were staged as N1 and 4.2% had distant metastases (M1). Capsular invasion and extrathyroidal expansion (ETE) were present in 14.4% and 29.3% of the cases, respectively. Multifocality was observed in 50.3% of all the cases; 4.2% of cases were familial, defined as cases with two first- or second-degree relatives also affected with PTC. The average age for controls was 43.8 ± 14.2 years, and the female:male ratio was 3:1 (*n* = 1077 [76.1%] vs. *n* = 339 [23.9%], respectively). The majority of the controls were Caucasian (93.8%; Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy).

TABLE 1. PATIENTS CHARACTERISTICS

Characteristic	PTC (n = 1216)	PTC clinicopathologic subtypes		
		cPTC (n = 891)	fvPTC (n = 243)	microPTC (n = 328)
Average age at diagnosis, <i>M</i> ± <i>SD</i>	41.3 ± 14.5	41.3 ± 14.7	39.9 ± 13.3	44.0 ± 13.0
Sex				
Female	935 (76.9%)	680 (76.3%)	194 (79.8%)	274 (83.5%)
Male	281 (23.1%)	211 (23.7%)	49 (20.2%)	54 (16.5%)
Ethnicity				
Afro-American	42 (3.5%)	26 (2.9%)	13 (5.4%)	8 (2.4%)
Asian	30 (2.5%)	26 (2.9%)	3 (1.2%)	5 (1.5%)
Caucasian	1144 (94.1%)	839 (94.2%)	227 (93.4%)	315 (96.0%)
T stage				
T0	2 (0.2%)	0 (0.0%)	1 (0.4%)	2 (0.6%)
T1	638 (54.3%)	501 (58.3%)	112 (47.5%)	314 (95.7%)
T2	263 (22.4%)	171 (19.9%)	69 (29.2%)	1 (0.3%)
T3	218 (18.6%)	150 (17.5%)	46 (19.5%)	10 (3.1%)
T4	53 (4.5%)	37 (4.3%)	8 (3.4%)	1 (0.3%)
N stage				
N0	722 (61.6%)	500 (58.3%)	186 (78.8%)	243 (75.0%)
N1	450 (38.4%)	357 (41.7%)	50 (21.2%)	81 (25.0%)
M stage				
M0	1132 (95.9%)	835 (96.5%)	222 (94.1%)	321 (98.5%)
M1	49 (4.2%)	30 (3.5%)	14 (5.93%)	5 (1.53%)
Multifocality				
No	582 (49.70%)	415 (48.59%)	130 (55.08%)	181 (55.86%)
Yes	589 (50.30%)	439 (51.41%)	106 (44.9%)	143 (44.1%)
Lymphovascular invasion				
No	954 (83.0%)	707 (84.1%)	189 (82.2%)	301 (94.1%)
Yes	195 (17.0%)	134 (15.9%)	41 (17.8%)	19 (5.9%)
Capsular invasion				
No	988 (85.6%)	740 (87.2%)	191 (84.1%)	301 (94.1%)
Yes	166 (14.4%)	109 (12.8%)	36 (15.9%)	19 (5.9%)
Extrathyroidal expansion				
No	441 (70.7%)	338 (70.3%)	75 (75.8%)	183 (92.4%)
Yes	183 (29.3%)	143 (29.7%)	24 (24.2%)	15 (7.6%)
Positive family history ^a				
No	1165 (95.8%)	848 (95.2%)	239 (98.4%)	311 (94.8%)
Yes	51 (4.2%)	43 (4.8%)	4 (1.7%)	17 (5.2%)

^aPositive family history defined as cases with two first- or second-degree relatives also affected with PTC.

PTC, papillary thyroid carcinoma; cPTC, classic PTC; fvPTC, follicular variant PTC; microPTC, microcarcinomas.

Association of GWAS variants with PTC in an Ohio population

All five GWAS markers showed strong association with PTC risk, displaying similar ORs to those that were reported in the original studies and several follow-up publications (Table 2). In a previous publication from 2013, the association between these GWAS markers and PTC risk in the Ohio population was already confirmed. The 1216 cases analyzed in the present study include the 620 cases described in the 2013 report (26). Likewise, of the 1416 controls in the present study, 805 were part of the previous study. This current study differs from the prior analysis of PTC risk because associations with clinical variables were not previously analyzed. In the present study, the strongest association between a risk allele and the development of PTC was detected for rs965513 (9q22) located close to *FOXE1* (odds ratio [OR]=1.81, $p=8.20 \times 10^{-24}$), and the weakest for rs944289 (14q13) located 3.2 kb from *PTCSC3*, 89 kb from *MBIP*, and 337 kb from *NKX2-1* (OR=1.23, $p=0.000489$). In order to determine whether there are differences in the association with risk dependent on histological and clinical subtypes of PTC, the association with cPTC ($n=891$), fvPTC ($n=243$), and microPTC ($n=328$) were analyzed separately (Supplementary Table S2A–C). The risk for cPTC carried by the variants rs944289 and rs116909374 located at 14q13 was slightly lower when compared with all the cases (OR=1.19, $p=0.00584$ and OR=1.76, $p=0.000264$ vs. OR=1.23, $p=0.000489$ and OR=1.90, $p=0.00000754$, respectively). Interestingly, the same variants carried higher risk for fvPTC (OR=1.31, $p=0.00950$ and OR=2.88, $p=0.000000730$, respectively), while rs965513 showed a weaker predisposition for fvPTC (OR=1.62, $p=0.00000290$). Two variants (rs2439302 and rs966423) showed only a trend toward significant association with fvPTC ($p=0.170$ and $p=0.257$, respectively). All the markers displayed significant association with microPTC, with risks similar to those observed for all cases.

Association between individual germline variants and clinical parameters of PTC

Several associations were observed between the germline variants and clinical/pathological features that are listed in Tables 3 and 4. The SNP rs965513 located at 9q22 close to *FOXE1* was associated with larger tumor size at the time of

diagnosis (regression coefficient 0.14, $p=0.025$) and ETE (OR=1.29, $p=0.045$). The variant rs2439302 located at 8p12 was associated with higher risk of multifocality (OR=1.24, $p=0.012$) and N1 status (OR=1.24, $p=0.016$). Interestingly, the risk allele of SNP rs116909374 was protective against lymph node metastases (OR=0.61, $p=0.016$). Several non-statistically significant trends toward associations were also detected. The variant rs116909374 showed borderline association with multifocal status (OR=1.39, $p=0.073$) and, consistent with its protective association with N status, a trend toward protective action against lymphovascular invasion (OR=0.61, $p=0.081$). Rs965513 showed borderline association with lymphovascular invasion (OR=1.23, $p=0.066$).

Association between combined germline variants and clinical parameters of PTC

Since the genetic predisposition to PTC is likely polygenic, this study analyzed whether the risk alleles of the tested markers might be accumulated in individuals with a given clinical feature. In order to accomplish this analysis, CGRS and wCGRS were calculated for selected markers, as described in the Methods. The results of the analysis are presented in Tables 3 and 4.

There was significant accumulation of the risk alleles of all five markers with multifocality (CGRS OR=1.12, $p=0.008$; wCGRS OR=1.09, $p=0.045$). There was a trend toward significance for the tumor diameter and ETE with wCGRS (regression coefficient 0.06, $p=0.09$, and regression coefficient 1.13, $p=0.075$, respectively), but it was not significant for CGRS (regression coefficient 0.03, $p=0.396$, and regression coefficient 1.08, $p=0.249$, respectively) likely due to the fact that all the association was coming from one variant (rs965513; Table 4).

In order to determine whether low-risk PTC patients might differ genetically from high-risk patients, the tested population was divided into two groups: very low-risk (T1a, N0, M0) and high-risk (T3–T4 or M1) patients, and then the potential differences in risk allele distribution were analyzed between the groups. As seen in Table 3, there was no significant association for the number of risk alleles of all five SNPs between low- and high-risk patients (CGRS: OR=1.01, $p=0.907$; wCGRS: OR=1.02, $p=0.758$), consistent with similar genetic risk in both groups of patients.

TABLE 2. THE ASSOCIATION RESULTS FOR THE GWAS VARIANTS WITH PTC

SNP	Locus	Frequency		OR [CI]	p-Value
		Cases (n=1216)	Controls (n=1416)		
rs965513[A]	9q22.33	0.47	0.33	1.81 [1.62–2.04]	8.20×10^{-24}
rs944289[T]	14q13.3	0.62	0.57	1.23 [1.09–1.38]	0.000489
rs116909374[T]	14q13.3	0.06	0.03	1.90 [1.44–2.53]	0.00000754
rs2439302[G]	8p12	0.52	0.45	1.30 [1.16–1.45]	0.00000511
rs966423[C]	2q35	0.48	0.42	1.27 [1.14–1.42]	0.0000218

GWAS, genome-wide association studies; OR, odds ratio; CI, confidence interval.

TABLE 3. ASSOCIATION BETWEEN GERMLINE VARIANTS AND CLINICAL PARAMETERS OF PTC (N= 1216)

SNP	Locus	Sex (males)		Age		T0-T2 vs. T3-T4		N stage		M stage		High risk vs. low-risk patients	
		OR [CI]	p-Value	Slope	p-Value	OR [CI]	p-Value	OR [CI]	p-Value	OR [CI]	p-Value	OR [CI]	p-Value
rs965513	9q22.33	1.05 [0.87-1.26]	0.640	-0.01 [-1.16 to 1.14]	0.984	1.06 [0.88-1.29]	0.536	1.10 [0.93-1.31]	0.270	0.86 [0.56-1.30]	0.465	1.13 [0.91-1.41]	0.256
rs944289	14q13.3	1.03 [0.84-1.25]	0.800	-1.04 [-2.26 to 0.17]	0.092	1.05 [0.86-1.30]	0.623	0.99 [0.83-1.19]	0.942	0.76 [0.49-1.17]	0.212	1.00 [0.80-1.25]	0.999
rs116909374	14q13.3	0.88 [0.59-1.32]	0.510	0.19 [-2.33 to 2.72]	0.880	0.95 [0.61-1.44]	0.812	0.61 [0.41-0.90]	0.016	1.38 [0.57-2.87]	0.421	0.82 [0.51-1.29]	0.39
rs2439302	8p12	1.01 [0.83-1.22]	0.946	0.55 [-0.64 to 1.73]	0.365	1.07 [0.88-1.31]	0.505	1.24 [1.04-1.49]	0.016	1.20 [0.78-1.87]	0.408	1.10 [0.88-1.37]	0.419
rs966423	2q35	1.01 [0.84-1.21]	0.934	-0.32 [-1.44 to 0.81]	0.581	0.85 [0.70-1.04]	0.114	1.05 [0.88-1.25]	0.596	0.75 [0.48-1.14]	0.184	0.84 [0.67-1.05]	0.126
CGRS		1.02 [0.93-1.13]	0.660	-0.27 [-0.86 to 0.32]	0.370	1.01 [0.92-1.12]	0.830	1.06 [0.97-1.16]	0.176	0.95 [0.76-1.17]	0.626	1.01 [0.90-1.13]	0.907
wCGRS		1.03 [0.93-1.13]	0.619	-0.18 [-0.77 to 0.42]	0.559	1.02 [0.92-1.12]	0.748	1.03 [0.95-1.13]	0.471	0.98 [0.78-1.21]	0.823	1.02 [0.91-1.14]	0.758

The lower half of the table shows Cumulative Genetic Risk Scores (CGRS) and weighted CGRS for all five variants for a given clinical parameter. Low-risk patients = T1a, N0, M0. High-risk patients = T3, T4, or M1.

TABLE 4. ASSOCIATION BETWEEN GERMLINE VARIANTS AND CLINICAL PARAMETERS OF PTC (N= 1216)

SNP	Locus	Tumor diameter		Multifocality		Lymphovascular invasion		Capsular invasion		Extrathyroidal expansion		MACIS score	
		Slope	p-Value	OR [CI]	p-Value	OR [CI]	p-Value	OR [CI]	p-Value	OR [CI]	p-Value	Slope	p-Value
rs965513	9q22.33	0.14 [0.02-0.27]	0.025	1.00 [0.85-1.18]	0.972	1.23 [0.99-1.53]	0.066	1.02 [0.81-1.29]	0.845	1.29 [1.01-1.67]	0.045	0.03 [-0.06 to 0.12]	0.542
rs944289	14q13.3	-0.01 [-0.14 to 0.12]	0.900	1.07 [0.90-1.27]	0.462	0.98 [0.78-1.23]	0.843	1.04 [0.82-1.34]	0.734	0.87 [0.67-1.12]	0.28	-0.07 [-0.17 to 0.03]	0.165
rs116909374	14q13.3	-0.06 [-0.33 to 0.21]	0.660	1.39 [0.97-2.00]	0.073	0.61 [0.34-1.03]	0.081	1.41 [0.88-2.19]	0.139	1.19 [0.69-2.01]	0.528	0.03 [-0.17 to 0.23]	0.767
rs2439302	8p12	0 [-0.13 to 0.12]	0.960	1.24 [1.05-1.48]	0.012	1.11 [0.88-1.40]	0.365	1.17 [0.92-1.5]	0.197	1.07 [0.83-1.38]	0.601	0.03 [-0.07 to 0.12]	0.567
rs966423	2q35	-0.09 [-0.21 to 0.04]	0.178	1.12 [0.95-1.32]	0.188	0.92 [0.74-1.15]	0.487	0.91 [0.72-1.14]	0.403	0.97 [0.75-1.25]	0.825	-0.05 [-0.14 to 0.04]	0.288
CGRS		0.03 [-0.04 to 0.09]	0.396	1.12 [1.03-1.22]	0.008	1.06 [0.95-1.18]	0.335	1.06 [0.94-1.20]	0.314	1.08 [0.95-1.23]	0.249	0 [-0.05 to 0.05]	0.925
wCGRS		0.06 [-0.01 to 0.12]	0.090	1.09 [1.00-1.19]	0.045	1.07 [0.96-1.20]	0.222	1.07 [0.95-1.21]	0.265	1.13 [0.99-1.28]	0.075	0.01 [-0.04 to 0.06]	0.636

The lower half of the table shows CGRS and wCGRS for all five variants for a given clinical parameter.

Association between germline variants and clinical parameters among histological PTC subtypes

In the cPTC group ($n=891$), an association was observed between rs965513 and tumor size (regression coefficient 0.20, $p=0.006$; Supplementary Table S3A and B). Notably, in the cPTC subtype, rs944289 displayed protective association with metastasis (M1 status: OR=0.58, $p=0.042$), despite the lack of this association in the general PTC group (OR=0.76, $p=0.212$). This may point to yet unknown molecular events in pathways involving the variant. There was a trend toward a significant association between rs116909374 and capsular invasion (OR=1.67, $p=0.060$).

In fvPTC ($n=243$), similar to the association detected in all the PTC cases, the risk allele of rs116909374 was associated with lymph node metastasis (N1 stage: OR=0.29, $p=0.049$). The risk alleles of the two variants that did not show association with fvPTC (rs2439302 and rs966432) were more frequent in older patients (regression coefficient 3.36, $p=0.007$, and slope 2.39, $p=0.048$, respectively; Supplementary Table S4A and B).

In the microPTC group, the variant rs2439302 showed association with ETE (OR=2.55, $p=0.032$), and SNP rs965513 showed a trend toward association with ETE (OR=2.01, $p=0.073$). The multivariate analysis showed borderline association with ETE (CGRS and wCGRS: OR=1.42, $p=0.068$, and OR=1.47, $p=0.042$, respectively). Similar to cPTC, rs116909374 showed a trend toward association with capsular invasion (OR=2.33, $p=0.090$; Supplementary Table S5A and B).

Association between gene expression and clinical factors

Seventy-three PTC paired tissue samples (unaffected and tumor tissue) were analyzed, and the expression of the genes

located adjacent to the tested variants (*PTCSC3*, *MBIP*, *NKX2-1*, *FOXO1*, *NRG1*, and *DIRC3*) was measured. For the *PTCSC2* expression analysis, 65 cases were tested due to unavailability of RNA from the rest of the patients. The expression of each gene was correlated with TNM staging, multifocality, and sex (Supplementary Figs. S1–S8). *DIRC3* did not show expression in unaffected thyroid tissue or PTC tissue. In unaffected thyroid tissue, the expression of the *MBIP* gene located at 14q13 (rs944289 and rs116909374) showed association with T stage ($p=0.010$). *NKX2-1* (14q13.3: rs944289 and rs116909374) displayed lower expression in PTC tissue in patients with N1 status ($p=0.040$). In males, the expression of *MBIP* and *PTCSC3* (14q13.3: rs944289 and rs116909374) in tumor tissue was significantly lower compared with that in females ($p=0.025$ and $p=0.036$, respectively; Fig. 1). No significant associations of clinical factors were observed within male and female groups for *MBIP* and *PTCSC3*. Some of the transcripts displayed borderline association. For instance, *NRG1* (8p12: rs2439302) expression showed borderline association with T stage in unaffected thyroid tissue ($p=0.062$; Supplementary Fig. S4A).

Discussion

PTC is associated with an indolent course of the disease. As a result, the mortality rate is low. The five-year survival rate for PTC is approximately 98% and is one of the most favorable among cancers (www.cancer.org). Although the mortality is low, residual or recurrent disease occurs in approximately 20–30% of cases (30). Assessing the risk of PTC recurrence may help in identifying patients needing more aggressive therapies and/or rigorous post-surgical follow-up. Several approaches have been described to assess the risk of more aggressive forms of thyroid cancer. One approach is to use molecular testing of PTC tumor tissue in search of mutations associated with a more aggressive course of the disease. This type of personalized

FIG. 1. Association between gene expression and clinical parameters. (A) *MBIP* expression showed significant association with T stage in unaffected thyroid tissue ($p=0.010$). (B) *NKX2-1* showed lower expression in patients with N1 status in papillary thyroid carcinoma tissue ($p=0.040$). (C) *MBIP* showed lower expression in males compared with females in tumor tissue ($p=0.025$). (D) *PTCSC3* displayed lower expression in males compared with females in tumor tissue ($p=0.036$).

medicine attempts to assist physicians in devising individualized management plans for each patient based on their mutation profile. Several reports have shown that a somatic mutation in *BRAF*^{V600E} is associated with a higher recurrence rate in PTC and may be useful in predicting the risk of metastases. In a recent publication, 1510 patients with thyroid cancer (97% had PTC) underwent routine molecular testing for *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARG* alterations with postsurgical follow-up tracking metastases and recurrence of the disease (31). It was shown that *BRAF*^{V600E}-positive PTC cases have a higher metastatic rate, while *RAS*-positive cases are predominately fvPTC with infrequent ETE and lymph node metastasis. The presence of both *BRAF*^{V600E} and the recently uncovered *TERT*^{C228T} mutation has been associated with a more aggressive type of PTC (24,32,33), suggesting a role for multi-gene testing in PTC in order to identify high-risk patients. Existing data on the effects of somatic mutations are contrasted by the paucity of data regarding correlations between germline heritable variants and clinicopathologic features of PTC, despite its high familiarity.

The present study aimed to analyze the association between well-established GWAS risk variants and clinicopathologic features in PTC. Additionally, the study searched for associations between expression levels of target or adjacent genes with clinical outcome. In calculating the association between the five-marker variants and PTC risk, the findings were clearly in line with previous data (5,6,26,34,35). This was to some degree expected, in part because some of the previous evidence was obtained using subsets of the present cases ($n=620$) and controls ($n=805$). However, with the present larger numbers of cases ($n=1216$) and controls ($n=1416$), the previous findings were not only confirmed but also strengthened in terms of statistical significance. Moreover, in the present study, it was possible to perform association studies separately for the histological subtypes of PTC. Perhaps most notably, all markers carried different degrees of predisposition to fvPTC compared with the entire PTC series. These differences may well be explained by future findings allowing the SNP marker alleles to be assigned to metabolic pathways and functional events.

Regarding the main findings, associations between germline variants and larger tumor size ($p=0.025$), higher multifocality ($p=0.012$), lymph node metastases ($p=0.016$), and ETE ($p=0.045$) have been uncovered. Overall, the effects of individual marker alleles on clinicopathologic parameters are relatively subtle, but this is expected, since the risks for developing PTC carried by those markers are low to intermediate. Larger tumor size is one of the conventional parameters used to predict cancer progression, and ETE is a risk factor for PTC recurrence (36,37). The present findings showing an association between rs965513 and tumor size and ETE suggest that germline variants may play a role not only as an “ignition” responsible for malignant transformation, but also may contribute to tumor growth and its invasion potential. The role of multifocality as a risk factor for a more severe clinical course is controversial. However, several reports have suggested multifocality status as a poor prognostic factor (38,39). In the present analysis of individual SNPs, only one variant (rs2439302) displayed significant association with multifocality. However, the effect of all five SNPs was additive, as reflected by CGRS ($p=0.008$). These data give hope that with the introduction of new markers over time, the resolution of the genetic/clinicopathologic associations will improve and eventually attain a level that allows their clinical use.

Unexpectedly, rs116909374 and rs944289 were associated with reduced frequency of lymph node metastases ($OR=0.61$, $p=0.016$) and distant metastases ($OR=0.58$, $p=0.042$), respectively. The same variants also displayed a higher OR for fvPTC compared with cPTC. Of note, fvPTC was recently reported to be associated with infrequent ETE and lymph node metastases (31). This finding coincides with the data showing stronger association of rs116909374 and rs944289 with fvPTC and, at the same time, a negative association with lymph node and distant metastases. That these two markers show identical association is worthy of some attention because they are located close to each other in the 14q13.3 thyroid-related locus (5,6). Clearly, it is possible or even likely that they participate in shared (presently unknown) underlying metabolic processes.

A potentially interesting association has been found between rs2439302 and ETE in the microPTC group ($OR=2.55$, $p=0.032$). This finding could be of major importance. However, there were only 15 (7.6%) ETE positive cases within the microPTC cohort. Further studies on large microPTC populations should address the question of whether this association is true.

Genetic variants predispose to cancers by impacting genes involved in important tumor specific pathways. The data showing an association between *MBIP* expression and T stage might draw attention to this gene as a putative transcript involved in PTC tumor growth. Interestingly, *MBIP* and *PTCSC3* showed stronger suppression in tumor tissue in males than it did in females. It is speculated that these predisposing genes might be related to the distinctive 3:1 female:male ratio in PTC. *NKX2-1* is an important gene in thyroid organogenesis (40). The lower expression of *NKX2-1* in tumor tissue in patients with N1 status might be an indicator of progression of dedifferentiation that could be one of the factors allowing tumors to disseminate (41).

Very recently, the results of two studies analyzing clinical correlations of the same GWAS SNPs that were studied here were published. In a Chinese study ($n=838$ PTC cases), the rs966423 SNP showed association with ETE and more advanced T stages (42). This association was not seen in the present population ($p=0.825$). In a large study from Poland analyzing 1836 cases with differentiated thyroid tumors, increased mortality was associated with rs966423. Curiously, it was the non-risk allele [T] that conferred higher mortality. Moreover, the risk was only significant when comparing [TT] homozygotes with carriers of the combined [TC] and [CC] genotypes (43). While these findings are difficult to interpret in comparison with those of the present study, it may be tentatively concluded that they are consistent with germline variants having an impact on clinical variables. More detailed, larger, perhaps multi-ethnic studies are obviously needed to elucidate these questions and contradictions.

In summary, by analysis of a large population of cases and controls, the associations of the studied GWAS variants with PTC are reconfirmed. This study shows that these variants carry variable risk for different histological subtypes of PTC. Significant associations were detected with tumor size and ETE (rs965513), tumor multifocality (rs2439302), and lymph node metastases (rs2439302). The accumulation of the risk alleles of all five GWAS variants was higher and statistically significant for multifocality. This study also shows that the expression of putative target genes of the tested variants is associated with T and N stages, and sex. The

findings link germline SNPs with phenotypes of PTC, and suggest potential usefulness of germline genetic markers in predicting the clinical course of PTC.

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Author Disclosure Statement

The authors have nothing to disclose.

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