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ORIGINAL ARTICLE

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Targeted next-generation sequencing in papillary thyroid carcinoma patients looking for germline variants predisposing to the disease

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Abstract

Purposess The purpose of this study was using next-generation sequencing technique to explore the potential association between germline variants of 14 targeted genes and papillary thyroid carcinoma (PTC) predisposition as well as disease progression.

Methods In all, 516 subjects were enrolled in this study including 416 PTC patients and 100 healthy controls. PTC patients were divided into distant metastasis group and non-distant metastasis group. Patients in distant metastasis group were further divided into radioiodine-refractory PTC (RR-PTC) and non-RR-PTC depending on their response to radioiodine therapy. Genomic DNA was extracted from peripheral blood sample and MiSeq Benchtop Sequencer was used for sequencing.

Results We found rs11246050 in *NLRP6* (dominant model, OR/95% CI: 2.028/1.091–3.769, $p = 0.025$), rs2286742 and rs3740530 in *HABP2* (recessive model, OR/95% CI: 9.644/1.307–71.16, $p = 0.026$ and 3.989/1.413–11.26, $p = 0.009$), rs2736098 in *TERT* (recessive model, OR/95% CI: 2.322/1.028–5.242. $p = 0.042$) and rs62054619 in *GAS8-AS1* (recessive model, OR/95% CI: 2.219/1.067–4.617, $p = 0.033$) were associated with the risk of PTC. rs1137282 in *KRAS* (dominant model, OR/95% CI: 0.5430/0.3192–0.9236, $p = 0.024$), rs1347591 and rs4461062 in *NUP93* (dominant model, OR/95% CI: 0.6121/0.4128–0.9076, $p = 0.015$ and 0.6156/0.4157–0.9117, $p = 0.015$) were associated with low risk of distant metastatic disease in PTC patients. rs33954691 in *TERT* was associated with the risk of RR-PTC under dominant model (OR/95% CI: 3.161/1.596–6.262).

Conclusions Germline variants of related genes could be associated with the susceptibility of PTC as well as disease progression (distant metastasis and radioiodine-refractory status). However, these results must be further verified and the potential biological functions of these germline variants in the pathogenesis of PTC remain to be determined in future studies.

Keywords Papillary thyroid carcinoma · Next-generation sequencing · Metastasis · Radioiodine-refractory PTC

Introduction

Papillary thyroid carcinoma (PTC) is considered as the major contribution to the worldwide increasing of overall thyroid cancer incidence. With relatively good prognosis, it is commonly treated as an indolent tumor. However, about 10% of PTC patients can develop distant metastatic disease (mostly in lungs and bones), which will largely lower patients' quality of life and increase disease specific mortality. Although conventional treatment strategy that adequate thyroidectomy followed by systemic radioiodine therapy and thyroid-stimulating hormone (TSH) inhibition shows a good disease control effect, about 20–40% of patients with distant metastatic disease will finally be diagnosed with radioiodine-refractory PTC (RR-PTC) [1–3].

The etiology of PTC is not clearly characterized. Both environmental and genetic factors can contribute to its tumorigenesis and progression. Radiation exposure to the thyroid gland is the most commonly recognized risk fact related to PTC. Meanwhile, this kind of tumor shows a high degree of heritability. Several genome-wide association studies (GWAS) have been performed to identify genetic

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predisposition to PTC [4–7]. Genes like *FOXE1*, *NKX2-1*, *DIRC3*, and *NRG1* are frequently reported and they are confirmed to be related to thyroid cancer risk among Asian and European populations [8–12]. Recently, a heterozygous germline missense mutation, *HABP2* G534E (rs7080536), was found as a causative mutation in familial nonmedullary thyroid carcinoma (FNMTC). In vitro studies demonstrated that HABP2 had a tumor-suppressive effect, while the G534E variant resulted in loss of function [13]. Afterwards, this variant had drawn much attention among researchers in this field. Following works have been done to try to verify this new finding. But except one study [14], others were not able to confirm the association between *HABP2* G534E variant and familial/sporadic nonmedullary thyroid carcinoma predisposition [15–22].

Aberrant activation of MAPK signaling pathway by genetic alterations (mutations in *BRAF*, *RAS*, *TERT* promoter, etc.) plays a central role in the carcinogenesis and progression of differentiated thyroid cancer. Among them, $BRAF^{V600E}$ is well known as the risk mutation for PTC, which occurs in 29–83% of patients [23]. This somatic mutation in *BRAF* is also found very important in the prognosis prediction and risk classification of PTC patients [24]. By now, only a few studies have focused on the contribution of the germline variants of genes in MAPK pathway to PTC predisposition and prognosis. Xing reported that $BRAF^{V600E}$ was not a germline mutation or susceptibility genetic event for FNMTC [25]. Zhang et al. investigated the potential association between the germline *BRAF* variants (four single-nucleotide polymorphisms) and PTC based on a case-control study and they found *BRAF* SNP variants rs17161747, rs1042179, and rs3748093 could predispose to PTC in those with a family history of cancer, smokers, and both those of age < 45 years and nondrinkers, respectively [23].

In this study, we use next-generation sequencing (NGS) technique to explore the potential association between germline variants of 14 targeted genes (*BRAF*, *KRAS*, *NRAS*, *HRAS*, *TERT*, *HABP2*, *EIF1AX*, *PPM1D*, *CHEK2*, *S100A7*, *NLRP6*, *NUP93*, *GAS8-AS1*, and *LPAR4*) and PTC predisposition as well as disease progression.

Materials and methods

Subjects

PTC patients and healthy controls were enrolled in a single institution from January 2014 to December 2015. All patients were sporadic PTCs. This study was approved by the ethics committee of our institution and written informed consent was achieved from each patient (for patient whose age was younger than 18 years, written informed consent was obtained from his/her statutory guardian additionally).

Healthy control group

Inclusion criteria for volunteer healthy controls: (1) normal thyroid ultrasound examination (no nodule detected by high-frequency ultrasound examination); and (2) normal thyroid function in blood test (including free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone, thyroglobulin (Tg), anti-Tg antibody (TgAb), anti-thyroid peroxidase antibody).

Exclusion criteria: (1) history of thyroid disease; (2) history of tumor (benign and malignant); and (3) presence of familiarity for thyroid carcinoma.

PTC patient group

All patients had pathologically proven conventional PTC after near/total thyroidectomy and were referred to our Department for Radioiodine Therapy. Before ^{131}I treatment, patients were asked to withdraw thyroid hormone medication for 3–4 weeks and begin a low iodine diet for 2 weeks. Routine patient work-up performed 1 day before radioiodine administration included measurements of FT3, FT4, TSH, Tg, and TgAb, neck ultrasonography and other examinations like chest CT scan if necessary. An empirical treatment dose of ^{131}I was administered (30–100 mCi for residual thyroid ablation, 100–150 mCi for lymph node metastases and 150–200 mCi for distant metastases) under serum TSH level ≥ 30 mIU/L. After ^{131}I treatment, patients were divided into two different groups according to their therapeutic ^{131}I scintigraphy (whole body planar scan and/or SPECT/CT imaging) and stimulating Tg level (TSH ≥ 30 mIU/L) which was tested before radioiodine therapy.

Distant metastasis group

Inclusion criteria: (1) ^{131}I scintigraphy showed distant metastases; (2) or metastatic lesions found in other imaging modalities like CT, MRI, or ^{18}F-FDG PET/CT scan even without iodine uptake in ^{131}I scintigraphy; and (3) serum sTg > 10 ng/mL; (4) or distant metastasis confirmed by pathology.

Exclusion criteria: (1) other malignancy history and (2) no evidence of distant metastatic disease.

Patients in distant metastasis group were further divided into RR-PTC and non-RR-PTC. Criteria for RR-PTC were as following: (1) metastatic lesions do not show radioiodine uptake even after successful remnant thyroid ablation; (2) metastatic lesions lose the ability to take up radioiodine; (3) radioiodine uptake retained in some lesions but not in

others; and (4) disease progresses despite substantial uptake of radioiodine.

Non-distant metastasis group

Patients those not qualified for distant metastasis group were included in the non-distant metastasis group.

DNA extraction and sequencing

Two milliliters of peripheral blood sample from each eligible subject was collected and used for genomic DNA isolation. For PTC patients, all samples were collected before radioiodine therapy under TSH stimulating. DNA extraction was performed by following the manufacturer's standard procedure using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). After purification and quality control test, multiplex PCR enrichment was performed on an ABI 2720 Thermal Cycler (Life Technologies Corporation, USA) with following cycling program: 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 63–0.5 °C per cycle for 40 s, 72 °C for 1 min; 24 cycles of 94 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min; 72 °C for 2 min. Primers were designed using FastTarget Primer software (V5.0.1, Genesky, Shanghai) covering all the coding sequences and most of the untranslated regions. The PCR products of each sample were labeled with 8 bp index and all the libraries of each sample were pooled. After cluster generation and hybridization of sequencing primer, sequencing was carried out by using MiSeq Benchtop Sequencer (Illumina) in one single lane following the manufacturer's protocol.

NGS data analysis

Sequencing reads were aligned to hg19 using the Burrows–Wheeler Aligner (BWA) [26]. SNV calling was performed using both GATK [27] and VarScan programs [28]. The Annovar program was used for SNV annotation [29]. The functional effect of non-synonymous SNVs was evaluated by the PolyPhen-2, SIFT, and MutationTaster [30–32]. Non-synonymous SNVs with SIFT score of <0.05, Polyphen-2 score of >0.85 or MutationTaster score of >0.85 were considered as significant of not being benign.

Statistics analysis

Subjects' clinicopathologic data were analyzed by using the Statistical Package for the Social Sciences, version 20.0 (SPSS, Chicago, IL, USA). Student's *t*-test (assuming equal variances) or Welch-test (assuming unequal variances) and Chi-square statistic test were used for comparison. For the genetic association study, age and gender adjusted analysis was performed both in dominant (the low-frequency allele is regarded as dominant allele) and recessive (the low-frequency allele is regarded as recessive allele) inheritance models by PLINK [33]. For details of the dominant and recessive models, please visit the official website of PLINK (http://zzz.bwh.harvard.edu/plink/anal.shtml#model). A *P*-value of <0.05 was considered statistically significant.

Results

Subjects

In all, 516 subjects were enrolled in this study including 416 PTC patients and 100 healthy controls. Basic characteristics of all the subjects can be found in Table 1. No significant differences of age and gender were found between PTC patient group and healthy control group. Of the 416 PTC patients, 185 had distant metastatic disease and 231 had non-distant metastatic disease. Of the 185 patients with distant metastasis, 53 patients finally diagnosed with RR-PTC (Fig. 1).

Low-frequency variants (frequency < 0.01)

For low-frequency single nucleotide variants analysis, only those with high single nucleotide polymorphism (SNP) calling quality were used. In all, a total of 32-point mutations in 9 genes were found in 62 subjects (51 PTC patients and 11 healthy controls) (Table 2). Mutations in *NLRP6*, *PPM1D*, and *KRAS* were only detected in PTC patients group. In all, 23 mutations were only found in PTC patients group. Of them, rs138864377 in *HABP2* and rs144756946 in *TERT* were the most frequent ones ($N = 6$). Sanger sequencing was performed in these 12 subjects and the result ascertained the existence of these two mutations (rs138864377 in *HABP2* and rs144756946 in *TERT*).

Common-frequency variants (frequency ≥ 0.01)

In all, we found 49 common-frequency single nucleotide polymorphisms in 12 genes. rs62487918, rs41282721, rs2736098, and rs74265472 polymorphisms were deleted for further analysis because these SNPs deviated significantly from Hardy–Weinberg equilibrium in healthy control group. The summary of common-frequency variants findings is demonstrated in Table 3.

PTC patients vs. healthy control

Logistic analysis showed that rs11246050 in *NLRP6* (dominant model, OR/95% CI: 2.028/1.091–3.769, $p = 0.025$), rs2286742 and rs3740530 in *HABP2* (recessive model, OR/95% CI: 9.644/1.307–71.16, $p = 0.026$ and 3.989/1.413–11.26, $p = 0.009$), rs2736098 in *TERT*

Table 1 Basic clinicopathologic characteristics

	Control $N = 100$	PTC $N = 416$	*P*-value	non-DM $N = 231$	DM $N = 185$	*P*-value
Age/years	19–60	11–82	0.801	17–72	11–82	0.849
Mean/SD	42.5/10.5	42.7/13.2		42.8/11.9	42.6/14.7	
Gender (*N*/%)						
Male	40/40	154/37.0	0.581	78/33.8	76/41.1	0.125
Female	60/60	262/63.0		153/66.2	109/58.9	
T stage (N/%)			–			0.001
T1	–	234/56.3		159/68.8	75/40.5	
T2	–	85/20.4		29/12.6	56/30.3	
T3	–	41/9.9		25/10.8	16/8.6	
T4	–	56/13.5		18/7.8	38/20.5	
Maximum diameter (cm, mean/SD)	–	2.1/1.1		1.7/0.9	2.5/1.2	0.001
N stage (*N*/%)			–			0.067
N0	–	27/6.5		20/8.7	7/3.8	
N1	–	386/92.8		209/90.5	177/95.7	
Nx	–	3/0.7		2/0.9	1/0.5	
Stage (*N*/%)						0.001
I	–	216/51.9		216/93.5	0/0.0	
II	–	151/36.3		10/4.3	141/76.2	
III	–	5/1.2		5/2.2	0/0.0	
IV	–	44/10.6		0/0.0	44/23.8	
Tumor foci (*N*/%)			–			0.939
Unifocal	–	192/46.2		107/46.3	85/45.9	
Multifocal	–	224/53.8		124/53.7	100/54.1	
sTg(ng/mL)						0.001
Median	–	28.0		5.7	177.4	
Range		0.04–21799		0.04–531.5	14–21799	
TgAb(IU/mL)						0.470
Median	–	16.7		17.9	15.6	
Range		10–4000		10–4000	10–4000	

PTC papillary thyroid carcinoma, *non-DM* non-distant metastasis, *DM* distant metastasis, *sTg* stimulating thyroglobulin, *TgAb* anti-Tg antibody

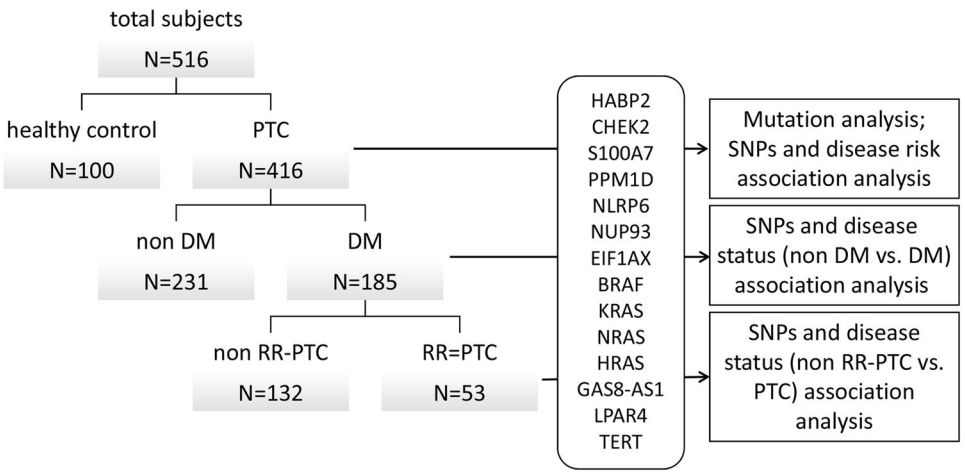

Fig. 1 Outline of patient grouping and study design. Next-generation sequencing technique was used to explore the potential association between germline variants of 14 targeted genes and PTC predisposition as well as disease progression. In all, 516 subjects were enrolled in this study including 416 PTC patients and 100 healthy controls. PTC papillary thyroid carcinoma, DM distant metastasis, RR-PTC radioiodine-refractory papillary thyroid carcinoma, SNP single nucleotide polymorphism

Table 2 Mutation summary (low-frequency variant)

SNV no.	Gene	SNP ID	Ref allele	Alt allele	Predicted protein variants	Gene region	Function	PTC	Control
1	HABP2	rs138864377	G	A	Exon9:c.947G>A:p.G316E	Exonic	NS	6	0
2	TERT	rs144756946	G	A	Exon2:c.1138C>T:p.P380S	Exonic	NS	6	0
3	CHEK2	rs587781379	C	T	Exon6:c.755G>A:p.S252N	Exonic	NS	3	0
4	CHEK2	–	C	T	Exon4:c.452G>A:p.G151D	Exonic	NS	3	0
5	PPM1D	rs140786757	C	T	Exon6:c.1550C>T:p.T517I	Exonic	NS	2	0
6	NLRP6	rs751415614	G	A	Exon2:c.146G>A:p.R49H	Exonic	NS	2	0
7	NLRP6	rs772775833	C	T	Exon6:c.2366C>T:p.T789M	Exonic	NS	2	0
8	BRAF	rs397507459	CGGCGC	–	Exon1:c.95_100del:p.32_34del	Exonic	NFD	2	0
9	HABP2	–	T	C	Exon1:c.47T>C:p.L16P	Exonic	NS	1	0
10	HABP2	rs542838125	G	T	Exon3:c.223G>T:p.D75Y	Exonic	NS	1	0
11	HABP2	rs141433300	C	T	Exon12:c.1384C>T:p.R462C	Exonic	NS	1	0
12	NUP93	rs750817566	G	A	Exon18:c.1948G>A:p.V650I	Exonic	NS	1	0
13	PPM1D	rs200809297	G	A	Exon6:c.1742G>A:p.R581Q	Exonic	NS	1	0
14	KRAS	–	T	C	Exon2:c.77A>G:p.N26S	Exonic	NS	1	0
15	LPAR4	rs767232879	A	G	Exon5:c.445A>G:p.I149V	Exonic	NS	1	0
16	LPAR4	–	A	G	Exon5:c.643A>G:p.I215V	Exonic	NS	1	0
17	NLRP6	–	C	T	Exon4:c.451C>T:p.L151F	Exonic	NS	1	0
18	NLRP6	rs201891372	C	T	Exon7:c.2534C>T:p.T845I	Exonic	NS	1	0
19	CHEK2	rs202051128	T	C	Exon10:c.1032A>G:p.I344M	Exonic	NS	1	0
20	CHEK2	rs121908701	C	T	Exon4:c.542G>A:p.R181H	Exonic	NS	1	0
21	CHEK2	–	C	–	Exon4:c.533delG:p.G178fs	Exonic	FD	1	0
22	CHEK2	–	T	C	Exon5:c.445–2A>G	Splicing	–	1	0
23	CHEK2	–	C	A	Exon2:c.3G>T:p.M1I	Exonic	NS	1	0
24	CHEK2	rs77130927	G	A	Exon4:c.538C>T:p.R180C	Exonic	NS	5	1
25	NUP93	rs561638940	G	A	Exon18:c.1985G>A:p.R662K	Exonic	NS	2	1
26	CHEK2	rs531398630	G	A	Exon11:c.1111C>T:p.H371Y	Exonic	NS	7	3
27	HABP2	rs778038877	G	C	Exon12:c.1518+1G>C	Splicing	–	0	1
28	NUP93	–	G	C	Exon14:c.1639G>C:p.A547P	Exonic	NS	0	1
29	LPAR4	–	A	T	Exon5:c.490A>T:p.I164F	Exonic	NS	0	1
30	TERT	rs201927653	G	A	Exon4:c.1931C>T:p.T644M	Exonic	NS	0	1
31	BRAF	rs397515331	–	CGGCGC	Exon1:c.100_101insGCGCCG:p.A34delinsGAA	Exonic	NFD	0	1
32	NUP93	rs771065608	C	T	Exon14:c.1570C>T:p.R524W	Exonic	NS	0	2

SNV single nucleotide variant,*SNP* single nucleotide polymorphism, *PTC* papillary thyroid carcinoma, *NS* nonsynonymous; *NFD* nonframeshift deletion, *FD* frameshift deletion

(recessive model, OR/95% CI: 2.322/1.028–5.242. $p = 0.042$) and rs62054619 in *GAS8-AS1* (recessive model, OR/95% CI: 2.219/1.067–4.617, $p = 0.033$) were associated with the risk of PTC. Interestingly, *HABP2* G534E variant (rs7080536) was not found in any of the subjects.

Distant metastasis vs. non-distant metastasis

rs1137282 in *KRAS* (dominant model, OR/95% CI: 0.5430/0.3192–0.9236, $p = 0.024$), rs1347591 and rs4461062 in *NUP93* (dominant model, OR/95% CI: 0.6121/0.4128–0.9076, $p = 0.015$ and 0.6156/0.4157–0.9117, $p = 0.015$) were found associated with low risk of distant metastatic disease in PTC patients.

RR-PTC vs. non-RR-PTC

rs33954691 in *TERT* was found associated with the risk of RR-PTC under dominant model (OR/95%CI: 3.161/1.596–6.262).

Discussion

In this study, we investigated the association between germline variants of 14 targeted genes and sporadic PTC predisposition as well as disease progression. For low-frequency variants, rs138864377 in *HABP2* and rs144756946 in *TERT* were the most frequent ones found in PTC patient group. For common-frequency variants, rs11246050 in *NLRP6*, rs2286742 and rs3740530 in *HABP2*, rs2736098 in *TERT*, and rs62054619 in *GAS8-AS1* were found might related to PTC predisposition. Further, we found rs1137282 in *KRAS*, rs1347591 and rs4461062 in *NUP93* might be associated with low risk of distant metastatic disease in PTC patients. We also found rs33954691 in *TERT* could be associated with the risk of RR-PTC.

HABP2 as a tumor-suppressor gene was first reported in 2015. The authors found that *G534E* variant conferred susceptibility to NMFTC and this variant could be found in 4.7% of 423 patients with thyroid cancer in TCGA database [13]. Following study from Zhang and Xing reported that

Table 3 Common-variant summary

	SNP	Model	Genotype	Case	Control	OR (95% CI)	*P*-value
PTC patients vs. healthy control	rs11246050 (NLRP6)	Dominant	G/G	273	57	2.028(1.091–3.769)	**0.025**
			G/A–A/A	136	14		
		Recessive	G/G–G/A	399	71	2.875e + 08(0–inf)	0.998
			A/A	10	0		
	rs2286742 (HABP2)	Dominant	A/A	308	80	1.435(0.8306–2.481)	0.195
			A/G–G/G	105	19		
		Recessive	A/A–A/G	376	98	9.644(1.307–71.16)	**0.026**
			G/G	37	1		
	rs2736098 (TERT)	Dominant	C/C	150	37	1.046(0.6646–1.647)	0.845
			C/T–T/T	263	62		
		Recessive	C/C–C/T	351	92	2.322(1.028–5.242)	**0.042**
			T/T	62	7		
	rs3740530 (HABP2)	Dominant	T/T	174	53	1.555(1.003–2.411)	0.050
			T/C–C/C	240	47		
		Recessive	T/T–T/C	355	96	3.989(1.413–11.26)	**0.009**
			C/C	59	4		
	rs62054619 (GAS8-AS1)	Dominant	A/A	139	36	1.173(0.7338–1.873)	0.506
			A/G–G/G	249	55		
		Recessive	A/A–A/G	312	82	2.219(1.067–4.617)	**0.033**
			G/G	76	9		
Distant metastasis vs. non-distant metastasis	rs1137282 (KRAS)	Dominant	A/A	160	181	0.5430(0.3192–0.9236)	**0.024**
			A/G–G/G	24	50		
		Recessive	A/A–A/G	182	231	2.05e + 09(0–inf)	0.999
			G/G	2	0		
	rs1347591 (NUP93)	Dominant	G/G	113	113	0.6121(0.4128–0.9076)	**0.015**
			G/A–A/A	71	116		
		Recessive	G/G–G/A	170	210	0.9102(0.4433–1.869)	0.798
			A/A	14	19		
	rs4461062 (NUP93)	Dominant	T/T	112	113	0.6156(0.4157–0.9117)	**0.015**
			T/C–C/C	72	118		
		Recessive	T/T–T/C	169	211	0.9364(0.4653–1.884)	0.854
			C/C	15	20		
RR-PTC vs. non-RR-PTC	rs33954691 (TERT)	Dominant	G/G	27	99	3.161(1.596–6.262)	**0.001**
			G/A–A/A	25	29		
		Recessive	G/G–G/A	50	125	1.667(0.2703–10.27)	0.582
			A/A	2	3		

SNP single nucleotide polymorphism, *OR* odd risk, *CI* confidence interval, *PTC* papillary thyroid carcinoma, *RR-PTC* radioiodine-refractory papillary thyroid carcinoma

The bold values mean $p < 0.05$

the prevalence of *HABP2* G534E was 14.0% of PTC patients from 29 kindreds and they concluded that *HABP2* G534E could be a susceptibility gene in a subgroup of FNMTC [14]. But unfortunately, after that, all the other following studies from different regions, which tried to verify the association between *HABP2* G534E and thyroid cancer could not give positive results. Although in the current study we did not find this variant (*HABP2* G534E) in any of our subjects which was consistent with another Chinese population-based report [34], we did find three other variants (rs138864377, rs2286742, and rs3740530) in *HABP2* might relate to the risk of PTC. Although conflict remains in this field, studies are needed to further unveil the true value of *HABP2* in thyroid cancer.

NLRP6, nod-like receptor pyrin domain-containing protein 6, is known to be related to inflammation and host

defense against microorganisms [35]. The concept that chronic inflammation could act as a pre-cancer condition was proposed for a long time and it has been verified by lots of clinical and epidemiological evidence. Specifically, the impact of inflammation in the development of papillary thyroid cancer is well-accepted. Here, we found *NLRP6* rs11246050 could be related to the predisposition of PTC. The biological function of *NLRP6* in human is not clearly elucidated. NLRP6 can participate in inflammasome formation and can involve in nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling regulation in myeloid cells [35]. As *NLRP6* is closely related to inflammation and the concept that chronic inflammation can be a pre-cancer condition, the question goes to that is *NLRP6* related to carcinogenesis? Study has reported that the absence of NLRP6 could accelerate colitis-associated tumor growth in mice [36], which provide the evidence that this protein may have anti-tumor effect. Recently, Wang et al. found that NLRP6 was down-regulated in about 75% of primary gastric cancer cases and low expression of NLRP6 was significantly associated with lymph node metastasis and poor overall survival [37]. Our current finding may indicate that this gene could be associated with thyroid cancer. And we hypothesize that this gene may act as a tumor suppressor gene in thyroid cancer although further studies are needed to verify this finding and unveil its potential role in thyroid tumorigenesis.

Somatic variants in *BRAF*, *RAS*, and *TERT* promoter are frequently found related to thyroid cancer risk and associated with patients' prognosis. However, germline variants of these genes in thyroid cancer patients are not clearly studied. Previous study has reported that *TERT* rs2736100 was significantly associated with elevated PTC risk in Chinese population [38]. Here we found another variant of *TERT*, rs2736098, might be associated with PTC risk. This variant was reported to be related to lots of cancers, but conflict remains. A recent meta-analysis showed that *TERT* rs2736098 polymorphism was associated with the risk of cancer in overall analysis [39]. Further studies are warranted to explicit the association between rs2736098 and thyroid cancer risk. Interestingly, we also found that *TERT* rs33954691 was associated with the risk of RR-PTC. Atzmon et al. reported that rs33954691 was associated with longevity. Except this information, little is known about this variant of *TERT* [40]. Radioiodine-refractory status of differentiated thyroid carcinoma is the largest obstacle in the treatment of this disease. Timely diagnosis of radioiodine-refractory status can be valuable to the patients by avoiding unnecessarily radioiodine therapy thus reducing the side effects of radiation. Although based on relatively small number of subjects and larger case-control studies are needed to further verify the result, our finding could provide essential information contributing to better management of these patients. Functional studies focused on the relationship between *TERT rs33954691* variant and the expression level of sodium–iodine symporter should be performed in the future.

Somatic mutations in *GAS8-AS1* might serve as a novel driver alternation in PTC. Further functional study found this long non-coding RNA (lncRNA) GAS8-AS1 act as a novel tumor suppressor in PTC through ATG5-mediated autophagy [41, 42]. Our results suggested that germline variant in *GAS8-AS1* might also associate with the risk of PTC. Considering the results from our study and previous publications, this gene could act as a tumor suppressor gene in the development of thyroid neoplasm. However, detailed functional studies are still warranted to verify this hypothesis. The *KRAS* rs12427141 might contribute to the susceptibility to PTC reported in one previous study [43]. However, this SNP was not detected in our cohort population and instead, we found that rs1137282 in *KRAS* might associate with low risk of distant metastatic disease in PTC patients. It was reported that somatic mutation in *NUP93* could be one of driver mutations in metastatic breast cancer [44]. Our current study firstly reported that germline variants in this gene might associate with the status of metastatic disease in PTC patients. It can be hypothesized that these variants in *KRAS* and *NUP93* could result in loss of function of these genes and then reduce the invasion and/or migration capabilities of the tumor cells.

Limitations must be mentioned. Firstly, selective bias can be existing in this study, because all the PTC patients included were referred to receive radioiodine therapy. Moreover, not all genes that implicated in PTC were included (e.g. SRGAP1) for analysis. Secondly, the number of healthy control subjects was relatively too small to analyze rare, as well as common variants of all the targeted genes. Additionally, for the disease status genetic association analysis, the number of patients in distant metastasis and RR-PTC groups were too small to derive a confirm result. Thirdly, diagnosis of distant metastasis and RR-PTC were mostly based on patients' medical history, medical imaging, serum Tg, and follow-ups. Histological evidence was not achieved in all patients. Fourthly, none of functional analysis was performed in the current study to further elucidate the bio-function of related genes in the development and progression of PTC. Our future work will focus on this topic. Finally, based on above, well-designed case-control studies with larger sample size from different region and races are warranted to further verify our findings.

In conclusion, germline variants of related genes could be associated with the susceptibility of papillary thyroid cancer and disease progression (distant metastasis and radioiodine-refractory status). Our findings could contribute

to better understanding of thyroid cancer tumorigenesis and development and therefor provide new strategies to better management of the patients. However, these results must be further verified and the potential biological functions of these germline variants in the pathogenesis of PTC remain to be determined in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethics committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Informed consent Written informed consent was achieved from each subject (for subject whose age was younger than 18 years, written informed consent was obtained from his/her statutory guardian additionally).

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