Mutations of the *TERT* promoter are associated with aggressiveness and recurrence/distant metastasis of papillary thyroid carcinoma

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Abstract. Several previous studies have shown that mutations in B-Raf proto-oncogene (*BRAF*) and telomerase reverse transcriptase (*TERT*) can be used for the diagnosis and prognosis of papillary thyroid carcinoma (PTC). However, whether mutations in *BRAF* and the *TERT* promoter may improve the accurate identification and risk stratification of high-risk patients in the early stage of PTC remains unclear and requires further investigation. In the present study, mutations in *BRAF* and the *TERT* promoter were examined in 205 patients using PCR and Sanger DNA sequencing. The potential association between mutations in these two genes and the clinicopathological characteristics of patients with PTC was then analyzed. *BRAF* mutations were identified in 169/205 (82.4%) patients, whereas only 8/205 (3.9%) patients presented mutations in the *TERT* promoter, seven patients exhibited a C228T mutation, and the remaining one had a C250T mutation. There were 6/205 (2.9%) patients with mutations in both *BRAF* and the *TERT* promoter. Importantly, compared with patients with no mutations, patients with mutations in *BRAF* were more likely to exhibit mutations in the *TERT* promoter. A significant difference in lymph node metastasis was found between the *BRAF* V600E mutation group and the group without mutations in *BRAF*. Mutations in the *TERT* promoter were significantly correlated with older age, extrathyroidal invasion, tumor multifocality and advanced tumor/node/metastasis stage, which are associated with the aggressiveness of PTC. Moreover, compared with patients exhibiting mutations in *BRAF*, mutations in the *TERT* promoter were found to be significantly associated with aggressive clinicopathological features and higher risk of recurrence or distant metastasis. Collectively, mutations in the *TERT* promoter were not frequent, but were significantly correlated with more aggressive clinicopathological features of PTC. Therefore, mutations in the *TERT* promoter may be an important factor in the genetic background of PTC, and detection of such mutations may help the accurate identification and management of high-risk patients with recurrent or distant metastasis.

Introduction

Papillary thyroid carcinoma (PTC) is a common endocrine malignant tumor, that has a high incidence worldwide (1,2). PTC usually develops slowly, and most patients with PTC have a high overall survival (3). However, ~10% of PTC cases are characterized by aggressive characteristics and high mortality rates (4-6). Recently, various studies have emphasized the importance of risk stratification in order to design individualized treatments for patients with aggressive PTC (7-9). Therefore, it is important to identify novel molecular biomarkers to improve the accurate identification of high-risk patients with early-stage PTC.

B-Raf proto-oncogene *(BRAF)*, a major human oncogene, has been identified in various cancers, including thyroid carcinoma (10,11). The mitogen-activated protein kinase (MAPK) signaling pathway may be activated by the *BRAF* V600E mutation and subsequently contribute to the tumorigenesis of thyroid cancer (12). Mutations in *BRAF* occur in ~50% of patients with PTC and have been reported to be associated with the aggressiveness-associated features of PTC, including older age, lymph node metastasis, larger tumor size and advanced tumor stage (13-16). However, contrasting results have been reported, and no significant associations between *BRAF*

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mutations and high-risk PTC characteristics were detected in several studies (17-19).

Human telomerase reverse transcriptase (*TERT)* is an important gene involved in the maintenance of chromosomal integrity and genome stability (20). *TERT* encodes the catalytic reverse transcriptase subunit of the telomerase enzyme (20). In total, two common mutations in the *TERT* promoter are located at positions -124 and -146 bp upstream of its translation start site, and are characterized by a C>T mutation at position 1,295,228 (C228T) and 1,295,250 of chromosome 5 (C250T), respectively. Mutations in the *TERT* promoter lead to *TERT* overexpression by creating an extra E26 binding motif, thus facilitating cancer growth (21,22). This novel genetic alteration occurs in PTC with a prevalence of 7.5-27% (23-26). Interestingly, mutations in *BRAF* and the *TERT* promoter could co-exist in PTC (25). Several studies have reported that mutations in the *TERT* promoter are associated with aggressive clinicopathological characteristics, especially when *BRAF* V600E mutations were also identified (27-29). However, another study found contrasting results (30). Therefore, the significance of *TERT* promoter mutations in predicting the aggressiveness of PTC is inconclusive and requires further investigation.

In the present study, the incidence and clinicopathological significance of *BRAF* and *TERT* promoter mutations were analyzed in patients with PTC. Additional studies on *TERT* and *BRAF* mutations may clarify whether these molecular factors could be used as biomarkers for the diagnosis and/or prognosis of patients with PTC.

Materials and methods

Patients and tissue samples. In the present study, 205 patients with PTC were enrolled at The Affiliated Yantai Yuhuangding Hospital of Qingdao University from January 2015 to December 2016. The surgical procedures for patients with PTC, including conventional papillary thyroid carcinoma (CPTC) and papillary thyroid microcarcinoma (PTMC), were based on the Guidelines for the diagnosis and treatment of thyroid nodules and differentiated thyroid cancer (31). According to the surgical procedure recommended in the guidelines, patients with thyroid papillary carcinoma underwent total thyroidectomy or thyroid gland combined with isthmus resection, and routine central lymph node dissection at the tumor site. Based on the preoperative and intraoperative conditions, it was decided whether to perform lymph node dissection of the contralateral neck area. In addition, the selection of routine use of radioactive iodine after surgery was also based on the aforementioned guidelines. According to the World Health Organization classification criteria, 118 patients were diagnosed with CPTC and 87 patients with PTMC. The American Joint Committee on Cancer staging system (32) was used for the classification of the TNM stage. After institutional review board approval and informed patient consent, thyroid tumor specimens were obtained for genetic analysis and clinicopathological data was retrospectively collected. All mutational analyses were performed after surgery, and the results had no influence on the surgical procedures. Patients who declined genetic testing or lacked clinicopathological data were excluded from the study. The clinicopathological data of the patients enrolled in the present study are presented in Table I.

Genomic DNA isolation. Genomic DNA in the formalin-fixed and paraffin-embedded specimens was extracted using a DNA Extraction kit (Promega Corporation) according to the manufacturer's instructions.

Mutational analysis of BRAF V600E and the TERT promoter. A human *BRAF* mutant gene detection kit (Amoy Diagnostics Co., Ltd.) was used for the detection of *BRAF* V600E mutation, as previously described (33). DNA was further analyzed using an ABI7500 real-time PCR thermocycler (Promega Corporation). The 5-carboxyfuorescein (FAM) and 5-hexachloro-fuorescein (HEX) contained in the *BRAF* mutant gene detection kit was used. The thermocycling conditions were as follows: 95˚C For 5 min, 15 cycles of 95˚C for 25 sec, 64˚C for 20 sec, 72˚C for 20 sec, and then 31 cycles of 93˚C for 25 sec, 60˚C for 35 sec, 72˚C for 20 sec. The primers used were the following: Forward, 5'-TCATAATGCTTGCTCTGATAG GA-3' and reverse, 5'-GGCCAAAAATTTAATCAGTGG A-3'. The mutation plot was determined by the cycle threshold values of FAM according to the manufacturer's instructions. The quality of the extracted DNA was verified by the amplification of a housekeeping gene, which was reported in the HEX channel. PCR was used to amplify the *TERT* promoter containing the C228T and C250T mutation hotpots, and the PCR products were sequenced for the detection of *TERT* promoter mutations, as previously reported (34,35). Taq polymerase was used and purchased from Kapa Biosystems; Roche Diagnostics. The primers for *TERT* promoter region were the following 5'-AGTGGATTCGCGGGCACAGA-3' (sense) and 5'-CAGCGCTGCCTGAAACTC-3' (antisense) and the PCR conditions were 95˚C for 3 min, followed by 10 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 68˚C for 1 min. This was then followed by 30 cycles of the same settings except for elongation for an additional 5 sec in each cycle. The PCR was completed with a final elongation step at 68˚C for 7 min.

Statistical analysis. Statistical analysis was performed using SPSS (version 19.0; IBM Corp.). Fisher's exact test and χ^2 tests were used for analyzing the relationship between mutations and clinicopathological features of patients with PTC. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Prevalence of mutations in BRAF and the TERT promoter in patients with PTC. Mutations in *BRAF* and the *TERT* promoter were investigated in 205 PTC patients (161 females and 44 males). Mutations in *BRAF* and the *TERT* promoter were analyzed with quantitative PCR and Sanger DNA sequencing, respectively (Fig. 1). In total, 169 patients exhibited *BRAF* V600E mutations, accounting for 82.4% (169/205) of patients with PTCs. Mutations of the *TERT* promoter were found in eight patients, with a prevalence of 3.9% (8/205; Table I). Of the eight cases analyzed, seven presented a C228T mutation (7/8) and the remaining patient exhibited a C250T mutation. According to previous studies, only one type of *TERT* promoter mutation is commonly found (34). No cases of

Table I. Association between *BRAF* V600E or *TERT* promoter mutations and clinicopathological characteristics in patients with papillary thyroid carcinoma.

	BRAF V600E				*TERT* promoter mutation			
Clinicopathological features	Mutated, n=169	Wild type, n=36	OR (95% CI)	P-value	Mutated, n=8	Wild type, n=197	OR (95% CI)	P-value
Sex								
Female	133	28	0.947	0.903	5	156	2.283	0.373
Male	36	8	(0.398-2.256)		3	41	(0.524-9.950)	
Age at diagnosis (years)								
≤45	90	16	0.702	0.337	1	105	7.989	0.030[a]
>45	79	20	(0.341-1.448)		7	92	(1.001-66.154)	
Tumor size (mm)								
≤10	74	12	0.642	0.248	1	85	5.312	0.142
>10	95	24	(0.301-1.368)		7	112	(0.641-44.002)	
Extrathyroidal invasion								
No	87	24	1.885	0.097	1	110	8.851	0.025[a]
Yes	82	12	(0.885-4.014)		7	87	(1.069-73.300)	
Multifocality								
Single	85	24	1.976	0.074	1	108	8.494	0.027[a]
Multifocal	84	12	(0.928-4.208)		7	89	(1.026-70.344)	
Lymph node metastasis								
No	69	22	2.277	0.026[a]	2	89	2.472	0.305
Yes	100	14	(1.090-4.759)		6	108	(0.487-12.551)	
TNM stage								
I-II	100	23	1.221	0.600	1	122	11.387	0.007[a]
III-IV	69	13	(0.579-2.574)		7	75	(1.374-94.385)	

[a]P<0.05. *BRAF*, B-Raf proto-oncogene; *TERT*, telomerase reverse transcriptase; OR, odds ratio; TNM, tumor/node/metastasis.

simultaneous mutations (C228T and C250T) were found in the present study. In addition, among the eight patients with *TERT* promoter mutations, six presented with the *BRAF* V600E mutation (6/8). In total, 118 patients with CPTC and 87 patients with PTMC was involved in the present study. The mutation prevalence of *BRAF* V600E and *TERT* promoter was different in these two histological subtypes. In patients with CPTC, the mutation rate of *BRAF* V600E and *TERT* promoter was 79.66 and 5.93%, while in patients with PTMC the mutation rate was 86.21 and 1.15% (data not shown).

Correlation between mutations in BRAF or the TERT promoter and clinicopathological features of PTC. In the present study, the association between mutations in *BRAF* or the *TERT* promoter and the clinicopathological parameters of PTC was investigated. As shown in Table I, a significant difference in lymph node metastasis was detected between patients with the *BRAF* V600E mutation and patients without *BRAF* mutations (P=0.026). However, no significant associations were observed between the *BRAF* V600E mutation and patient sex, age at diagnosis, tumor multifocality, extrathyroidal invasion or tumor/node/metastasis (TNM) stage. Compared with the group without mutations in the *TERT* promoter, mutations of the *TERT* promoter were significantly associated with an older age at diagnosis, tumor multifocality, extrathyroidal invasion and advanced TNM stage (P=0.03, P=0.027, P=0.025 and P=0.007, respectively), but not with tumor size, sex or lymph node metastasis.

In order to determine the significance of mutations in *BRAF* and the *TERT* promoter in risk stratification, patients with PTC were divided into the following three subgroups: i) Negative for mutations in both *BRAF* and the *TERT* promoter ($BRAF^{-}/TERT^{-}$); ii) only positive for the *BRAF* V600E mutation, ($BRAF^{+}/TERT^{-}$); and iii) with or without the *BRAF* V600E mutation and positive for *TERT* promoter mutations, ($BRAF^{+/-}/TERT^{+}$). Compared with the $BRAF^{-}/TERT^{-}$ group, the $BRAF^{+}/TERT^{-}$ group was significantly associated with tumor multifocality (P=0.042) and lymph node metastasis (P=0.012), while the $BRAF^{+/-}/TERT^{+}$ group showed a significant association with extrathyroidal invasion (P=0.004) and advanced TNM stage (P=0.013), and tumor multifocality (P=0.004), and trend towards an increase in lymph node metastasis (P=0.05; Table II). Interestingly, the $BRAF^{+/-}/TERT^{+}$ group showed significant association with extrathyroidal invasion (P=0.032) and TNM stage (P=0.009) in comparison with the $BRAF^{+}/TERT^{-}$ group (Table II). Importantly, the $BRAF^{+/-}/TERT^{+}$ group had a higher incidence of recurrence and distant metastasis compared with both $BRAF^{-}/TERT^{-}$ and

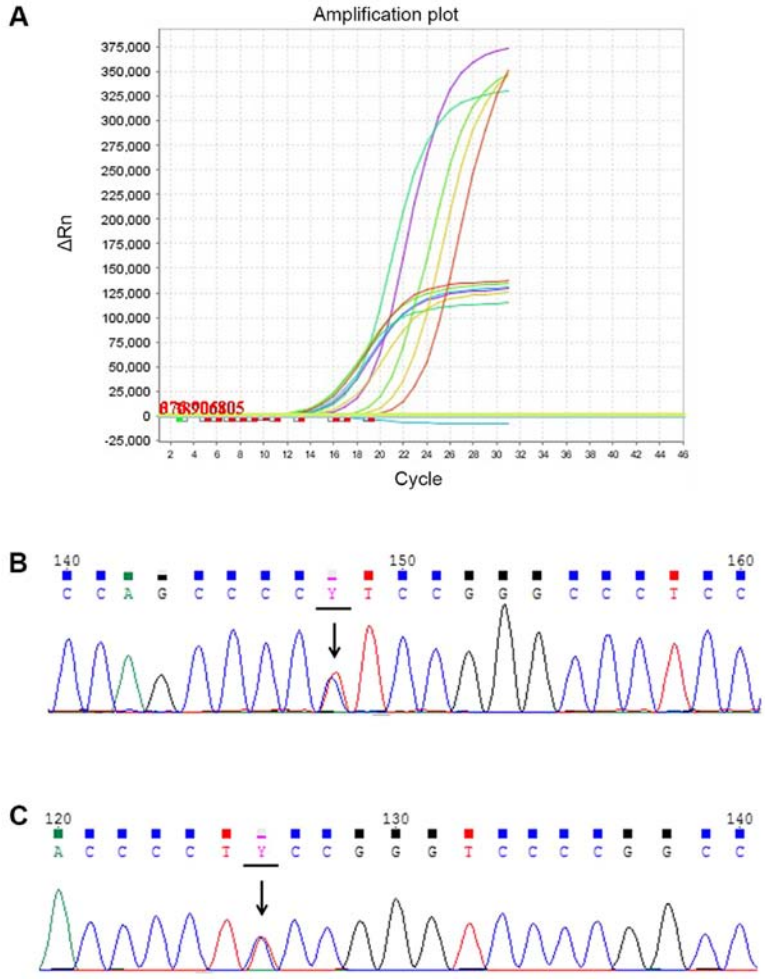

Figure 1. *BRAF* V600E and two common *TERT* promoter mutations in patients with PTC. (A) Quantitative PCR amplification plot of PTC with *BRAF* V600E mutation. The different colored lines are different samples. The mutation is indicated by the higher amplification plot and the housekeeping gene is indicated by the lower amplification plot. Positive control is indicated in red. Negative control is indicated in blue. (B) Sequencing chromatogram of the C228T *TERT* promoter mutation in a case of PTC. (C) Sequencing chromatogram of the C250T *TERT* promoter mutation in a case of PTC. Arrows indicate the mutation. PTC, papillary thyroid carcinoma; *BRAF*, B-Raf proto-oncogene; *TERT*, telomerase reverse transcriptase.

BRAF^{+}/*TERT*$^{-}$ groups (Table III). Collectively, these two molecular biomarkers, and in particular, mutations in the *TERT* promoter, may be useful for the identification and management of patients with poor outcome.

Discussion

In the present study, mutations in the *TERT* promoter showed a greater association with the aggressive clinicopathological features of PTC compared with the *BRAF* V600E mutation. Moreover, patients with *TERT* promoter mutations had a poorer outcome, as assessed by recurrence and distant metastasis rates.

The prevalence of the *BRAF* V600E mutation in the present study was 82.4%, which was relatively high compared with the average worldwide prevalence of ~45%. Several studies have shown that the prevalence of the *BRAF* V600E mutation in patients from Asian countries, including Japan, South Korea and China, is higher than that of Western countries (16,27,36,37). The present study found that the frequency of mutations in the *TERT* promoter was lower than that of *BRAF* mutations. These differences in the mutation frequency can be caused by various factors, including iodide intake, endocrine disruptors, analysis of ethnically diverse groups and environmental factors in certain geographical areas, such as radiation and increased exposure to asbestos amphibole fluoroedenite in the volcanic areas (38-41). Moreover, it has been reported that the prevalence of the *BRAF* V600E mutation is increasing in China (27). Another important reason is that the distribution of *BRAF* mutation is associated with the distinct histological subtypes of PTC (40,41). The present study included 118 patients with CPTC and 87 patients with PTMC. In a prior study, the distribution of mutations in *BRAF* and the *TERT* promoter displayed a clear subtype-related pattern (25). In the present study, the mutation prevalence of *BRAF* V600E in patients with CPTC and PTMC was 79.66 and 86.21%, respectively. In addition, the mutation rate of the *TERT* promoter was 5.93 and 1.15% in patients with CPTC and PTMC, respectively.

It has been reported in several previous studies that the *BRAF* V600E mutation is associated with high-risk clinicopathological characteristics (15,42-45). However, some studies have shown no significant association between *BRAF* V600E mutations and any clinicopathological features

Table II. Association of *BRAF* V600E/*TERT* promoter mutation status with clinicopathological characteristics in patients with papillary thyroid carcinoma.

Clinicopathological features	*BRAF*$^{-}$/*TERT*$^{-}$ (1) n=34	*BRAF*$^{+}$/*TERT*$^{-}$ (2) n=163	*BRAF*$^{+/-}$/*TERT*$^{+}$ (3) n=8	P-value	1 vs. 2		1 vs. 3		2 vs. 3	
					OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Sex										
Female	26	130	5	0.484	0.825 (0.342-1.988)	0.668	1.950 (0.380-10.013)	0.412	2.364 (0.537-10.399)	0.367
Male	8	33	3							
Age at diagnosis (years)										
≤45	16	89	1	0.056	0.739 (0.352-1.550)	0.423	6.222 (0.689-56.203)	0.114	8.419 (1.013-69.989)	0.028[a]
>45	18	74	7							
Tumor size (mm)										
≤10	12	73	1	0.135	0.672 (0.312-1.450)	0.309	3.818 (0.419-34.812)	0.398	5.678 (0.683-47.204)	0.140
>10	22	90	7							
Extrathyroidal invasion										
No	24	86	1	0.009[a]	2.149 (0.966-4.779)	0.057	16.800 (1.822-154.894)	0.004[a]	7.818 (1.041-64.987)	0.032[a]
Yes	10	77	7							
Multifocality										
Single	24	84	1	0.008[a]	2.257 (1.015-5.019)	0.042[a]	16.800 (1.822-154.894)	0.004[a]	7.443 (0.895-61.866)	0.064
Multifocal	10	79	7							
Lymph node metastasis										
No	22	67	2	0.022[a]	2.627 (1.217-5.670)	0.012[a]	5.500 (1.021-31.589)	0.050	2.094 (0.410-10.691)	0.303
Yes	12	96	6							
TNM stage										
I-II	23	99	1	0.015[a]	1.352 (0.617-2.961)	0.450	14.636 (1.598-134.097)	0.013[a]	10.828 (1.301-90.097)	0.009[a]
III-IV	11	64	7							

[a]P<0.05. *BRAF*, B-Raf proto-oncogene; *TERT*, telomerase reverse transcriptase; OR, odds ratio; TNM, tumor/node/metastasis.

Table III. Association between *BRAF* V600E/*TERT* promoter mutation status and recurrences or distant metastasis in patients with papillary thyroid carcinoma.

Mutation status	No. of recurrences or distant metastasis/no. patients	1 vs. 2		1 vs. 3		2 vs. 3	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
BRAF^{-}/*TERT*$^{-}$ (1)	1/34						
BRAF^{+}/*TERT*$^{-}$ (2)	4/169	0.800 (0.087-7.388)	1.000	19.800 (1.707-129.643)	0.018[a]	24.750 (4.338-141.205)	0.002[a]
BRAF$^{+/-}$/*TERT*$^{+}$ (3)	3/8						

[a]P<0.05. *BRAF*, B-Raf proto-oncogene; *TERT*, telomerase reverse transcriptase; OR, odds ratio.

of PTC (17,18,46-48). A modest association between *BRAF* V600E mutations and PTC clinicopathological features was reported in the present study. Due to the high prevalence of *BRAF* V600E, it is difficult to use this marker to improve the risk stratification and identify high-risk patients with poor outcome. Therefore, in addition to *BRAF* V600E, additional studies are required to identify novel gene mutations associated with aggressive PTC phenotypes.

In the present study, mutations in the *TERT* promoter were significantly associated with aggressive clinicopathological features compared with patients without mutations or the *BRAF* V600E mutation alone, such as the presence of extra-thyroidal invasion and advanced TNM stage. Furthermore, patients harboring *TERT* promoter mutations showed a higher possibility of recurrence and distant metastasis. The present data suggested that mutations in the *TERT* promoter may be a promising genetic molecular biomarker associated with aggressive PTC. The present findings are in line with previous studies, and suggest that mutations in the *TERT* promoter may enhance the aggressiveness of PTC (24,29,49). Mutations in *BRAF* and the *TERT* promoter co-existed in 6/8 patients with *BRAF* V600E in the present study. The *BRAF* V600E mutation may upregulate the expression of *TERT* by activating the MAPK pathway (25). Whether mutations in *BRAF* and *TERT* are directly related to PTC oncogenesis, and if these gene mutations have synergistic or additive effects on PTC, will require further investigation.

Genetic testing of thyroid cancer is of great significance for the diagnosis and prognosis assessment of patients with PTC and may facilitate follow-up treatments. Importantly, genetic testing can be used as an auxiliary means for pre-operative fine needle aspiration biopsy to diagnose unidentified thyroid nodules, thereby improving the accuracy of diagnosis (50). *BRAF* V600E plays an important role as a driving mutation in the early stage of tumorigenesis and has become an ideal biomarker for thyroid cancer (51). Moreover, *BRAF* V600E has been used as one of the prognostic indicators in patients with PTC in the 2015 edition of the American Thyroid Association (ATA) Guidelines (52). Previous studies have shown that *TERT* promoter mutation is involved in the pathogenesis of tumors and is associated with tumor aggressiveness (20). However, whether *TERT* promoter mutations could be used as a prognostic indicator for patients with PTC remains unclear, and the present study has contributed towards further understanding of this. The detection of *TERT* promoter mutations may be helpful to improve the ATA risk stratification system and guide clinicians to select appropriate treatments for patients with PTC.

One of the main limitations of the present study is that no follow-up data was obtained for the patients exhibiting these mutations, partly due to the short time period after the diagnosis and the better 5-year survival rate of PTC patients. In future studies, it would be useful to investigate whether *TERT* promoter mutations were of complementary value to the ATA risk stratification system, and could help identify the high-risk patients and guide appropriate treatments of them. In addition, the findings of the present study need to be further confirmed with a larger sample size, as the prevalence of *TERT* mutations was relatively low. Moreover, although *TERT* promoter mutations may be a promising molecular biomarker for identifying

aggressive PTC, the therapeutic potential of simultaneous mutations in *BRAF* and the *TERT* promoter requires further investigation.

In conclusion, mutations in the *TERT* promoter may have a low prevalence, but a high value in improving the risk stratification system and management of patients with aggressive PTC. The aggressiveness of PTC may be cooperatively driven by *TERT* promoter and other gene mutations, and the implication of *TERT* promoter mutations for the prognosis and treatment of patients with PTC should be further investigated.

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Availability of data and materials

The data used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

RL, YuL and CS conceived the study. RL, YuL, WC, JC, ZZ, LM, LC, HX, YZ, YoL, YX, QY and XY designed the experiments, provided reagents and collected data. ZZ conducted the experiments. YuL performed data analysis and wrote the manuscript. All authors read and approved the final version of the paper.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University. Written informed consent was provided by each patient. When the patient was <18 years of age, informed consent was provided by the patient's legal guardian.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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