TERT promoter mutations and long-term survival in patients with thyroid cancer

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Abstract

TERT promoter mutations are emerging prognostic biomarkers in multiple cancers and are found in highly aggressive thyroid cancer. Our aim is to investigate the prognostic value of these mutations for the outcome of thyroid cancer-related mortality in a large cohort of thyroid cancer patients. This was a retrospective study of 409 patients (393 with differentiated thyroid cancer) with a median age of 44 years (range 16–81 years) and median follow-up of 13 years (interquartile range 11–16 years). Analyses of associations between mutational status and various clinicopathological variables were performed. TERT promoter mutations were identified in 32 (9.8%) papillary, 11 (16.7%) follicular and seven (43.8%) poorly differentiated/anaplastic thyroid cancer patients. The presence of TERT promoter mutations was associated with factors such as increased age ($P < 0.001$), extrathyroidal invasion ($P = 0.01$), increased stage at diagnosis ($P < 0.001$) and dedifferentiated histological type ($P = 0.001$). A TERT promoter mutation was independently associated with poorer overall survival in patients with differentiated thyroid cancer (10-year survival rate, 66.2% vs 98.3% for wild type; adjusted HR, 7.18; 95% CI: 2.77–18.59) and in patients with papillary cancer (74.2% vs 99.3%; 14.20; 3.03–66.68). Concomitant TERT and BRAF mutations worsened the survival rate of patients with papillary cancer (82.6% vs 99.4% for exclusively BRAF mutation alone; 5.62; 1.85–17.09). In conclusion, the presence of TERT promoter mutations is independently associated with increased mortality in patients with differentiated thyroid cancer. The results suggest that inclusion of TERT promoter mutation analysis with conventional clinicopathological evaluation can lead to better prognostication and management for individual patients.

Key Words

- thyroid cancer
- TERT promoter
- BRAF
- prognosis
- mortality

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Introduction

Thyroid cancer is the most common endocrine malignancy, and its prevalence is increasing worldwide (Chen *et al.* 2009). In the USA, the 10-year overall survival rates for papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and poorly differentiated/anaplastic thyroid cancer (PDTC/ATC) are approximately 93, 85 and 14%, respectively (Hundahl *et al.* 1998). Given the increased likelihood of long-term survival in patients with differentiated thyroid cancer, it is important to distinguish patients who need aggressive treatment to reduce mortality from those who do not. Several clinicopathological prognostic variables have been used to stratify cancer patients, including increased age, histological type, tumor size, direct local tumor invasion, lymph node metastasis and distant metastasis (DeGroot *et al.* 1990, Mazzaferri & Kloos 2001). These conventional risk factors, alone or in combination as a component of staging system (Hay *et al.* 1993, Edge *et al.* 2010), imperfectly predict the final outcome of each individual patient.

Recently, molecular marker-based risk stratification of thyroid cancer has been proposed to better estimate the risk (Xing *et al.* 2013b). The v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) T1799A mutation has drawn much attention based on its high prevalence, occurring in about 45% of PTC and 25% of ATC cases and its association with PTC recurrence (Kim *et al.* 2012, Xing *et al.* 2015). However, many clinicians note that the mutation is common, whereas thyroid cancer-related mortality in patients with PTC is low. In a large multicenter study of 1849 patients with PTC, the *BRAF* T1799A mutation was associated with an increased cancer-related mortality, but it was no longer significant after the adjustment of risk factors, such as lymph node metastasis, extrathyroidal invasion and distant metastasis (Xing *et al.* 2013a).

In 2013, recurrent point mutations were found in the telomerase reverse transcriptase (*TERT*) promoter in 71% of melanoma genomes, and these somatic mutations enhanced promoter activity by two- to four-fold (Horn *et al.* 2013, Huang *et al.* 2013), which might immortalize proliferative cancer cells by maintaining telomere length (Vinagre *et al.* 2014). Starting with the seminal work of Liu and coworkers (2013), the importance of these findings has been reinforced in thyroid cancer by the association of mutations with tumor aggressiveness, early recurrence and even patient mortality (Melo *et al.* 2014, Xing *et al.* 2014a,b, Song *et al.* 2015). This potential prognostic marker is very promising, but its relationship to the long-term survival of patients with thyroid cancer requires

further study. Currently, none of the mortality risk systems takes molecular testing results into consideration. It is still unclear if patients with *TERT* promoter-mutated thyroid cancer should be managed differently than those with wild-type tumor. The goal of our study is to investigate the association of *TERT* promoter mutations with thyroid cancer-specific survival to lead to more informed decisions and better management of thyroid cancer.

Subjects and methods

Patients and clinicopathological data

The Institutional Review Board at Samsung Medical Center, Seoul, Korea, approved the study protocol and the use of human thyroid tissue. This study included 409 patients with thyroid cancer. All patients were treated with initial thyroidectomy (398 total or near-total thyroidectomy and 11 less than total thyroidectomy) and therapeutic neck dissection from October 1994 to December 2004. All patients received standard thyrotropin suppression, and 376 of them received radioiodine ablation as guided by standard criteria (Cooper *et al.* 2006, 2009). Sample characteristics obtained from medical records are presented in Table 1. Thyroid cancer samples were obtained from the tissue archives of the Department of Pathology of the Samsung Medical Center. Only one sample per individual was included. All mutational analyses were performed after surgical and radioiodine treatment of patients, and the test results did not affect the decision-making process of the acting physician. Thyroid cancer-related mortality, based on the date of registration of death where the primary cause of death was thyroid cancer, was obtained from the Korea National Statistical Office and hospital medical records.

DNA isolation from thyroid cancer samples

Tumor cells with a percentage greater than 75% were dissected under microscopy from 4 µm unstained sections by comparison with hematoxylin- and eosin-stained slides according to a standard protocol (Kim *et al.* 2014). Genomic DNA was extracted using a Qiagen DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.

Detection of *TERT* promoter mutations

Semi-nested polymerase chain reaction (PCR) was carried out to identify *TERT* promoter mutations

Table 1 Summary of clinical and genetic characteristics of 409 thyroid cancer patients.

Characteristic	N (%)
Sex	
Female	339 (82.9)
Male	70 (17.1)
Age (years)	
Median	44
Range	16–81
<45	226 (55.3)
≥45	183 (44.7)
TERT promoter mutations	
WT	359 (87.8)
228 C>T	46 (11.2)
250 C>T	4 (1.0)
BRAF mutation	
WT	121 (29.6)
1799 T>A	202 (49.4)
Missing data	86 (21.0)
Histological type	
PTC	327 (80.0)
FTC	66 (16.1)
PDTC/ATC	16 (3.9)
Multifocality	
Absent	299 (73.1)
Present	110 (26.9)
Lymph node metastasis	
Absent	206 (50.4)
Present	201 (49.1)
Missing data	2 (0.5)
Extrathyroidal invasion	
Absent	111 (27.1)
Present	248 (60.6)
Missing data	50 (12.2)
Distant metastasis	
Absent	384 (93.9)
Present	25 (6.1)
Stage at diagnosis ^a	
I	222 (54.3)
II	26 (6.4)
III	103 (25.2)
IV	58 (14.2)
Tumor size	
Median	2.8
Range	0.4–13.0
<2.0 cm	45 (11.0)
2.0–4.0 cm	302 (73.8)
>4.0 cm	62 (15.2)

BRAF, v-Raf murine sarcoma viral oncogene homolog B; FTC, follicular thyroid cancer; PDTC/ATC, poorly differentiated/anaplastic thyroid cancer; PTC, papillary thyroid cancer; TERT, telomerase reverse transcriptase.

^aStaging according to the American Joint Committee on Cancer (AJCC) Thyroid Cancer Staging System 2009 (Edge et al. 2010).

(Sohn et al. 2016). First-round PCR was performed using primers TERT-F and TERT-236-R as described previously (Liu et al. 2013). The 235bp-sized PCR amplicon was subjected to second-round PCR using primers TERT-F and TERT-163-R (Horn et al. 2013). The primer sequences for

TERT mutations are presented in Supplementary Table 1, see section on supplementary data given at the end of this article. PCRs were performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). Cycle sequencing was performed using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

Detection of BRAF T1799A mutation

Mutant enrichment with 3'-modified oligonucleotides-PCR (MEMO-PCR) and direct sequencing were performed as described previously (Lee et al. 2012). The obtained sequences were analyzed using the Sequencher program (Gene Codes Corp, Ann Arbor, MI, USA) and compared with a reference sequence (GenBank accession number NM_004333.4).

Target exome sequencing (including TERT promoter and BRAF)

Genomic DNA (250 ng) was sheared by Covaris S220 (Covaris, Woburn, MA, USA) and was used for the construction of a library using targeted exome sequencing (CancerSCAN) probes and SureSelect XT reagent kit, HSQ (Agilent Technologies) according to the manufacturers' protocols. CancerSCAN probes cover the TERT promoter region and whole exons of 381 genes, including BRAF. After enriched exome libraries were multiplexed, the libraries were sequenced on HiSeq 2500 sequencing platform (Illumina, San Diego, CA, USA). Briefly, a paired-end DNA sequencing library was prepared through genomic DNA shearing, end-repair, A-tailing, paired-end adaptor ligation and amplification. After hybridization of the library with bait sequences for 16 h, the captured library was purified and amplified with an index barcode tag, and the library quality and quantity were measured. Sequencing of the exome library was carried out using the 100 bp paired-end mode of the TruSeq Rapid PE Cluster kit and TruSeq Rapid SBS kit (Illumina).

Statistical analysis

We used univariate logistic regression analyses to evaluate the association of TERT promoter mutation status with conventional clinical and pathological variables (Table 2). We investigated the association between clinicopathological variables, TERT and BRAF mutation status with thyroid cancer-specific survival, defined as the time from the initial surgical treatment to thyroid

Table 2 Association of *TERT* mutation status with clinicopathological variables in 409 thyroid cancer patients.

Variables	<i>TERT</i> WT (N (%))	<i>TERT</i> mut (N (%))	Total	OR ^a (95% CI)	P value
Sex					
Female	297 (87.6)	42 (12.4)	339	1.00 (referent)	0.82
Male	62 (88.6)	8 (11.4)	70	0.91 (0.41–2.04)	
Age					
Per 5 year			409	1.96 (1.64–2.36)	<0.001
<45	222 (98.2)	4 (1.8)	226	1.00 (referent)	<0.001
≥45	137 (74.9)	46 (25.1)	183	18.64 (6.56–52.92)	
<i>BRAF</i> mutation					
WT	109 (90.1)	12 (9.9)	121	1.00 (referent)	0.43
Mutant	176 (87.1)	26 (12.9)	202	1.34 (0.65–2.77)	
Missing data	74 (86.0)	12 (14.0)	86		
Histological type					
PTC	295 (90.2)	32 (9.8)	327	1.00 (referent)	0.001
FTC	55 (83.3)	11 (16.7)	66	1.84 (0.88–3.88)	
PDTC/ATC	9 (56.3)	7 (43.8)	16	7.17 (2.50–20.55)	
Multifocality					
Absent	263 (88.0)	36 (12.0)	299	1.00 (referent)	0.85
Present	96 (87.3)	14 (12.7)	110	1.07 (0.55–2.06)	
Lymph node metastasis					
Absent	184 (89.3)	22 (10.7)	206	1.00 (referent)	0.39
Present	174 (86.6)	27 (13.4)	201	1.30 (0.71–2.36)	
Missing data	1 (50.0)	1 (50.0)	2		
Extrathyroidal invasion					
Absent	105 (94.6)	6 (5.4)	111	1.00 (referent)	0.01
Present	211 (85.1)	37 (14.9)	248	3.07 (1.26–7.50)	
Missing data	43 (86.0)	7 (14.0)	50		
Distant metastasis					
Absent	342 (89.1)	42 (10.9)	384	1.00 (referent)	0.003
Present	17 (68.0)	8 (32.0)	25	3.83 (1.56–9.42)	
Stage at diagnosis ^b					
I	216 (97.3)	6 (2.7)	222	1.00 (referent)	<0.001
II	25 (96.2)	1 (3.8)	26	1.44 (0.17–12.45)	
III	91 (88.3)	12 (11.7)	103	4.75 (1.73–13.04)	
IV	27 (46.6)	31 (53.4)	58	41.33 (15.80–108.10)	
Tumor size					
<2.0 cm	41 (91.1)	4 (8.9)	45	1.00 (referent)	0.08
2.0–4.0 cm	269 (89.1)	33 (10.9)	302	1.26 (0.42–3.73)	
>4.0 cm	49 (79.0)	13 (21.0)	62	2.72 (0.82–8.98)	

BRAF, v-Raf murine sarcoma viral oncogene homolog B; FTC, follicular thyroid cancer; PDTC/ATC, poorly differentiated/anaplastic thyroid cancer; PTC, papillary thyroid cancer; TERT, telomerase reverse transcriptase.

^aThe odds ratio (OR) represents the odds for being a *TERT* promoter mutation carrier as compared with a *TERT* promoter wild-type carrier;

^bStaging according to the American Joint Committee on Cancer (AJCC) Thyroid Cancer Staging System 2009 (Edge et al. 2010).

cancer-related mortality. Cases in which the end point was not reached at the time of the last follow-up were censored.

Survival analyses were focused on patients with differentiated thyroid cancer (PTC and FTC) because PDTC/ATC is uncommon and highly lethal. Univariate results were displayed using the Kaplan–Meier method, and hazard estimates and *P* values were calculated from Cox proportional hazard models. For univariate analyses of *BRAF* mutation, we used only cases with *BRAF* mutation status available. Because the effect of *BRAF* mutation was not significant in the univariate analyses, *BRAF* mutation was not included in multivariate analyses.

As ‘Stage at diagnosis’ is a composite variable, we decided to separate the variable into its components (‘Age’, ‘Extrathyroidal invasion’, ‘Distant metastasis’ and ‘Tumor size’) in some of the multivariate analyses. In the extended model, all main effects with univariate *P* values less than 0.20, except ‘Stage at diagnosis’, were evaluated simultaneously (Tables 3 and 4, model 1). In the restricted model, the ‘Stage at diagnosis’ variable replaced its components to avoid overfitting (Tables 3 and 4, model 2). The model diagnostic included both graphical and formal Schoenfeld residual checks of the proportional hazards assumptions (Harrell et al. 1996). We conducted

Table 3 Association of *TERT* mutation status and clinicopathological variables with thyroid cancer-specific survival in 393 differentiated thyroid cancer patients.

Variables	N ^a	10-year survival rate (%)	Univariate Cox models		Multivariate Cox model 1 ^b (extended model)		Multivariate Cox model 2 ^c (restricted model)	
			HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Sex								
Female	329	95.4	1.00 (referent)	0.31				
Male	64	92.0	1.60 (0.64–3.99)					
Age ^d								
Per 5 year	393		1.76 (1.60–1.92)	<0.001	1.51 (1.30–1.72)	<0.001		
<45	223	99.1	1.00 (referent)	<0.001				
≥45	170	89.3	17.23 (4.07–72.95)					
<i>TERT</i> mutations								
WT	350	98.3	1.00 (referent)	<0.001	1.00 (referent)	0.002	1.00 (referent)	<0.001
Mutant	43	66.2	23.26 (10.10–53.56)		5.19 (1.87–14.44)		7.18 (2.77–18.59)	
<i>BRAF</i> mutation								
WT	117	92.3	1.00 (referent)	0.28				
Mutant	199	97.5	0.60 (0.24–1.51)					
Histological type								
PTC	327	96.9	1.00 (referent)	0.002	1.00 (referent)	0.004	1.00 (referent)	<0.001
FTC	66	84.8	3.24 (1.47–7.15)		9.27 (2.06–41.72)		4.94 (2.09–11.68)	
Multifocality								
Absent	286	94.7	1.00 (referent)	0.98				
Present	107	95.3	0.99 (0.42–2.36)					
Lymph node metastasis								
Absent	199	94.4	1.00 (referent)	0.91				
Present	193	95.3	0.96 (0.44–2.10)					
Extrathyroidal invasion								
Absent	108	98.1	1.00 (referent)	0.11	1.00 (referent)	0.04		
Present	239	94.9	2.70 (0.79–9.22)		5.16 (1.06–24.99)			
Distant metastasis								
Absent	373	96.5	1.00 (referent)	<0.001	1.00 (referent)	0.23		
Present	20	65.0	8.66 (3.63–20.64)		1.89 (0.67–5.31)			
Stage at diagnosis ^e								
I	218	99.1	1.00 (referent)	<0.001			1.00 (referent)	0.003
II	26	92.3	8.18 (1.15–58.11)				4.58 (0.63–33.33)	
III	103	97.1	6.92 (1.40–34.32)				6.53 (1.26–33.84)	
IV	46	71.0	47.09 (10.81–205.09)				18.28 (3.58–93.25)	
Tumor size								
<2.0 cm	45	95.4	1.00 (referent)	0.03	1.00 (referent)	0.04		
2.0–4.0 cm	293	96.2	1.20 (0.28–5.23)		0.72 (0.16–3.37)			
>4.0 cm	55	87.0	3.58 (0.76–16.84)		2.52 (0.49–13.14)			

BRAF, v-Raf murine sarcoma viral oncogene homolog B; FTC, follicular thyroid cancer; PTC, papillary thyroid cancer; TERT, telomerase reverse transcriptase.

^aThe number of available data for a particular variable in the univariate analysis; ^bExtended model in which all predictors with univariate *P* values ≤0.20, except 'Stage at diagnosis', are included; no interactions were considered; ^cRestricted model that includes '*TERT* mutations', 'Histological type' and 'Stage at diagnosis'; no interactions were considered; ^dMultivariate Cox regression analysis results for 'Age' are analyzed and presented for the continuous linear variable; ^eStaging according to the American Joint Committee on Cancer (AJCC) Thyroid Cancer Staging System 2009 (Edge et al. 2010).

a sensitivity analysis by applying a backward elimination approach to a multivariate Cox regression including all the univariate explanatory variables.

In addition, we performed subgroup analysis for patients with PTC, the most common histological type. All *P* values were two sided, and a *P* value <0.05 was treated as statistically significant. All statistical analyses were performed using SPSS statistical software (version 21.0; SPSS).

Results

Clinical evaluation

In total, 421 thyroid cancer samples were screened for the study. The further analyzed cohort consisted of samples from 409 patients whose *TERT* promoter sequencing of tumor DNA was successful. There were 339 women and 70 men with a median age of 44 years (range 16–81 years) and a median follow-up duration of 13 years

Table 4 Association of *TERT* mutation status and clinicopathological variables with thyroid cancer-specific survival in 327 papillary thyroid cancer patients.

Variables	N ^a	10-year survival rate (%)	Univariate Cox models		Multivariate Cox model 1 ^b (extended model)		Multivariate Cox model 2 ^c (restricted model)	
			HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Sex								
Female	276	97.8	1.00 (referent)	0.07	1.00 (referent)	0.56	1.00 (referent)	0.21
Male	51	92.0	2.62 (0.91–7.56)		1.50 (0.38–5.99)		2.00 (0.68–5.84)	
Age ^d								
Per 5 year	327		1.74 (1.54–1.94)	<0.001	1.33 (1.06–1.59)	0.04		
<45	187	98.9	1.00 (referent)	0.002				
≥45	140	94.2	10.21 (2.32–44.99)					
<i>TERT</i> mutations								
WT	295	99.3	1.00 (referent)	<0.001	1.00 (referent)	0.001	1.00 (referent)	0.001
Mutant	32	74.2	33.38 (10.76–103.61)		10.78 (2.67–43.51)		14.20 (3.03–66.68)	
<i>BRAF</i> mutation								
WT	65	98.5	1.00 (referent)	0.28				
Mutant	199	97.5	3.12 (0.39–24.60)					
Multifocality								
Absent	228	97.3	1.00 (referent)	0.50				
Present	99	95.9	1.41 (0.51–3.89)					
Lymph node metastasis								
Absent	136	97.8	1.00 (referent)	0.48				
Present	190	96.3	1.47 (0.50–4.30)					
Extrathyroidal invasion								
Absent	73	100.0	1.00 (referent)	0.13	1.00 (referent)	0.27		
Present	233	95.7	4.85 (0.64–36.72)		3.45 (0.39–30.66)			
Distant metastasis								
Absent	314	97.4	1.00 (referent)	0.09	1.00 (referent)	0.78		
Present	13	84.6	3.66 (0.83–16.13)		1.32 (0.19–8.93)			
Stage at diagnosis ^e								
I	178	98.9	1.00 (referent)	<0.001			1.00 (referent)	0.64
II	17	100.0	NA				NA	
III	95	98.9	4.14 (0.76–22.62)				2.04 (0.31–13.35)	
IV	37	80.4	27.94 (6.12–127.64)				3.61 (0.47–27.47)	
Tumor size								
<2.0 cm	35	97.1	1.00 (referent)	0.20	1.00 (referent)	0.14		
2.0–4.0 cm	253	97.6	1.46 (0.19–11.32)		0.84 (0.10–6.82)			
>4.0 cm	39	92.1	3.91 (0.44–35.03)		3.37 (0.34–33.73)			

BRAF, v-Raf murine sarcoma viral oncogene homolog B; *TERT*, telomerase reverse transcriptase.

^aThe number of available data for a particular variable in the univariate analysis; ^bExtended model in which all predictors with univariate *P* values ≤ 0.20, except 'Stage at diagnosis', are included; no interactions were considered; ^cRestricted model that includes 'Sex', '*TERT* mutations' and 'Stage at diagnosis'; no interactions were considered; ^dMultivariate Cox regression analysis results for 'Age' are analyzed and presented for the continuous linear variable; ^eStaging according to the American Joint Committee on Cancer (AJCC) Thyroid Cancer Staging System 2009 (Edge et al. 2010).

(interquartile range 11–16 years). There were cumulative deaths in 5.5% (18/327) of PTC, 18.2% (12/66) of FTC and 75.0% (12/16) of PDTC/ATC patients. Most of these deaths were related to thyroid cancer. Specifically, 4.9% (16/327) of PTC, 15.2% (10/66) of FTC and 75.0% (12/16) of PDTC/ATC cases resulted in a thyroid cancer-related death. The baseline clinical data and mutation status of the tumors are presented in Table 1.

Mutational analysis

The *TERT* promoter was successfully PCR amplified and sequenced in 409 patients (327 PTC, 66 FTC and

16 PDTC/ATC). Recurrent mutations were located at previously described hotspots: chr.5:1,295,228C>T and chr.5:1,295,250C>T. The *TERT* promoter region showed mutant reads in 9.8% (32/327) of PTC, 16.7% (11/66) of FTC and 43.8% (7/16) of PDTC/ATC patients: the majority of the mutations were 228 C>T, and 250 C>T was found in three PTC and one FTC patients. Sequencing for *BRAF* was successful in 323 (79.0%) patients. The *BRAF* T1799A mutation was observed in 75.4% (199/264) of PTC, 0% (0/52) of FTC and 42.9% (3/7) of PDTC/ATC patients. For validation of the *TERT* promoter and *BRAF* genotyping results, we also performed high-coverage cancer panel sequencing in a randomly selected subset of *TERT*

promoter mutation-positive ($n=2$) and -negative cases ($n=8$). The results obtained by two different methods were identical in all selected cases (Supplementary Fig. 1A, B and Supplementary Table 2).

Association of *TERT* promoter mutation status with clinicopathological variables

TERT promoter mutations were significantly more common in older patients ($P<0.001$), in tumors with extrathyroidal invasion ($P=0.01$), in cases with distant metastasis ($P=0.003$) and with increased stage at diagnosis ($P<0.001$) (Table 2). The *TERT* mutation frequencies differed among histological type (9.8% in PTC, 16.7% in FTC and 43.8% in PDTC/ATC; $P=0.001$ for the frequency difference). Separate data for patients with differentiated thyroid cancer are presented in Supplementary Table 3. In patients with PTC, the largest subgroup, *TERT* promoter mutations were more common in the mutant *BRAF* than the wild type (12.1% (24/199) vs 3.1% (2/65); $P=0.03$).

Association of *TERT* promoter mutation status and clinicopathological variables with thyroid cancer-related mortality

Survival analyses were performed for patients with differentiated thyroid cancer (Fig. 1A and Table 3) and for patients with PTC (Fig. 1B and Table 4). In differentiated thyroid cancer patients (Table 3), univariate predictors of survival were *TERT* promoter mutation status ($P<0.001$), patient age ($P<0.001$), histological type (poorer in FTC compared with PTC; $P=0.002$), distant metastasis ($P<0.001$), stage at diagnosis ($P<0.001$) and tumor size ($P=0.03$). Patients with mutant *TERT* promoter tumors showed worse prognosis (10-year survival rate of 66.2%, compared with 98.3% for wild-type tumors). In multivariate analysis, however, the adjusted HRs (95% CI) were differently estimated according to variable selection strategy (5.19 (1.87–14.44), $P=0.002$ for the extended model; 7.18 (2.77–18.59), $P<0.001$ for the restricted model). In addition, patient age, histological type, extrathyroidal invasion, stage at diagnosis and tumor size were independent factors.

In patients with PTC (Table 4), factors that were significantly associated with poorer survival in univariate analyses included *TERT* promoter mutations ($P<0.001$), increased age ($P<0.001$) and increased stage at diagnosis (in particular stage IV, compared with lower stage; $P<0.001$). Multivariate analysis showed that *TERT*

Figure 1

Kaplan–Meier curves of thyroid cancer-specific survival by *TERT* mutational status. Results from the analysis of patients with (A) differentiated thyroid cancer and (B) papillary thyroid cancer. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0219>.

promoter mutations ($P=0.001$) and increased age ($P=0.04$) were independently associated with poorer survival. In the restricted model, the estimated adjusted HR (95% CI)

was 14.20 (3.03–66.68) for the mutant *TERT* promoter compared with the wild type, and stage at diagnosis was no longer a significant predictor of survival.

In a sensitivity analysis with backward elimination approach, the models included *TERT* mutations that are presented in Table 3 (*TERT* mutations with histological type and extrathyroidal invasion) and also in Table 4 (*TERT* mutations only). HRs for *TERT* mutations were 14.33 (4.81–42.71) and 39.47 (8.38–185.99) (Tables 3 and 4). Although these HRs differed from those in other models, they were contained within 95% CIs of HR in all other models; and their 95% CIs contained HRs in all other models.

For patients with PTC, we performed stratified analysis of four genotypes according to the *TERT* and *BRAF* mutational status. The Kaplan–Meier analysis (Fig. 2) shows significant difference in survival in relation to the genotypes (log-rank $P < 0.001$). There were no deceased cases in either wild type. The 10-year survival rates among the *BRAF* mutation only and concomitant *TERT* and *BRAF* mutations were 99.4% and 82.6%, respectively. *TERT* promoter mutations in addition to *BRAF* mutation significantly worsened PTC patient survival: when the genotypes replaced the “*TERT* mutations” variable in the extended and the

restricted models (Table 4), the adjusted HRs (95% CI) were 2.77 (1.15–6.65), $P = 0.02$ for the former model and 5.62 (1.85–17.09), $P = 0.002$ for the latter model.

Discussion

In this study, we found recurrent *TERT* promoter mutations in 9.8% of PTC, 16.7% of FTC and 43.8% of PDTC/ATC patients. In analyses of both differentiated thyroid cancer and PTC patients, the presence of *TERT* promoter mutations was found to be a marker of poor survival and remained significant on multivariate adjustment for conventional clinicopathological risk factors and for stage. The absence of the mutations conferred negligible mortality risk during long-term follow-up in PTC patients.

This study is notable because of the inclusion of a large cohort of thyroid cancer patients with robust clinicopathological data and adequate follow-up period (median 13 years), which is essential to evaluate the final outcome of a slowly progressing cancer with distinctively low mortality such as thyroid cancer (Kim et al. 2012). The results obtained here demonstrated that *TERT* promoter mutation status could be an independent prognostic marker in differentiated thyroid cancer for the outcome of long-term mortality, as suggested by preexisting studies (Landa et al. 2013, Liu et al. 2013, 2014a,b, Vinagre et al. 2013, Melo et al. 2014, Xing et al. 2014b). We observed that the point estimates for the HR for *TERT* varied across the models (i.e., extended, restricted and backward elimination based) as the HR was conditional on the variables selected in each model. Also, because mutant *TERT* is not very common, the HR was not estimated very precisely. Nevertheless, the independent effect of *TERT* mutations was demonstrated in all models, including the backward elimination-based model.

Cell immortalization is the step that is thought to be a prerequisite for the development of all human cancers; however, the exact mechanism of telomere stabilization had remained elusive. The discovery of *TERT* promoter mutations (-124C>T (C228T) and -146C>T (C250T)) (Horn et al. 2013, Huang et al. 2013) out of the non-coding region (so-called dark matter) of the human genome provided new mechanistic insight for telomere activation, particularly in tumors originating from tissues with a relatively low rate of self-renewal, such as glioma (Killela et al. 2013), bladder cancer (Hurst et al. 2014) and thyroid cancer (Liu et al. 2013). The mutations generate *de novo* consensus binding motifs for mitogen-activated protein kinase (MAPK)-dependent E26 transformation-specific (ETS) transcription factor (Strahl et al. 1996,

No. at risk

Both WT	63	63	59	21	0
<i>BRAF</i> MUT only	175	174	170	47	3
<i>TERT</i> MUT only	2	2	1	0	0
<i>TERT</i> and <i>BRAF</i> MUT	24	21	19	5	0

Figure 2

Kaplan–Meier curves of thyroid cancer-specific survival by four genotypes according to the *TERT* and *BRAF* mutational status. Results from the analysis of patients with papillary thyroid cancer. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0219>.

Horn *et al.* 2013, Huang *et al.* 2013). This mechanism is relevant in thyroid cancer as the majority of cases are driven by activation of the RAS-MAPK pathway via point mutations (*BRAF* and *RAS*) (The Cancer Genome Atlas Research Network 2014) or chromosomal rearrangements (*RET/PTC* (Nikiforov 2002), *AKAP9/BRAF* (Ciampi *et al.* 2005) and *TRK* (Russell *et al.* 2000)); therefore, it is expected that ETS transcription factors are highly activated. Mutant *TERT* promoters in thyroid cancer were responsible for the increased expression of the enzymatic subunit of the telomere holoenzyme (Muzza *et al.* 2015), resulting in the maintenance of chromosomal stability in proliferative cancer cells without control by apoptosis or senescence (Gunes & Rudolph 2013).

Most of the known associations among conventional clinicopathological variables and *TERT* promoter mutations were reproduced in this study, supporting our findings (Table 2 and Supplementary Table 3) (Liu *et al.* 2013, 2014a, Melo *et al.* 2014). In particular, the mutation frequency detected in differentiated thyroid cancers was about 11%, whereas the frequency increased to 44% in PDTC/ATC patients. As progressive dedifferentiation of PTC and FTC gives rise to PDTC and ATC, the close relationship between *TERT* promoter mutations and thyroid differentiation status may be one of the key biological mechanisms for the causal association between *TERT* promoter mutations and poor survival of patients even in cases morphologically classified as differentiated thyroid cancer. This relationship is consistent with *in vitro* evidence showing that the suppression of telomerase activity in melanoma cell lines induced cellular differentiation and reduced metastasis (Bagheri *et al.* 2006) or, vice-versa, the induction of differentiation leads to the downregulation of telomerase activity in immortalized cell lineages and embryonic stem cells (Sharma *et al.* 1995). In histological subgroup analysis of our data, survival differences based on *TERT* promoter mutational status were evident in PTC (Fig. 1B) and FTC (Supplementary Fig. 2A), but fell short of significance in already dedifferentiated cancers (Supplementary Fig. 2B). It is worth noting that the number of cases analyzed for dedifferentiated cancers was small compared with the number for PTC and FTC and that may be the reason for the lack of significance. A recent study of 106 patients with anaplastic thyroid cancer found an association of *TERT* C228T with *BRAF* T1799A mutations, older patient age and distant metastasis (Shi *et al.* 2015).

We observed that *TERT* promoter mutations were more common in PTC patients with the *BRAF* T1799A

mutation, as suggested by some previous reports (Liu *et al.* 2013, Melo *et al.* 2014), but not all (Landa *et al.* 2013, Muzza *et al.* 2015). An American study including 507 patients who were treated for PTC from 1990 to 2012 at Johns Hopkins Hospital reported that a combination of the *TERT* C228T and *BRAF* T1799A mutations was associated with a high risk of early recurrence (Xing *et al.* 2014b). On the contrary, a recent Italian study reported that neither *BRAF* nor *RAS* mutations had additional effects on disease persistence in patients with PTC and FTC (Muzza *et al.* 2015). However, the number of cases with coexisting mutations in the latter study was too small to reach statistically meaningful conclusion. In stratified analysis of our data of PTC, the vast majority of *TERT* promoter mutations occurred on a background of *BRAF* mutation. This coexistence significantly worsens PTC patient survival compared with having *BRAF* mutation alone, supporting the notion that the aggressiveness of *TERT* mutations is in fact largely from the synergy of coexisting genetic abnormalities that activate the RAS-MAPK pathway, particularly *BRAF* and *RAS* mutations (Ngeow & Eng 2014, Xing *et al.* 2014b, Song *et al.* 2016, Xing 2016). A previous large study on the role of *TERT* mutations on the mortality of PTC also supports this interaction (Xing *et al.* 2014a).

There are several limitations to this study. This was a retrospective study and was thus prone to selection bias. An independent prospectively collected cohort with long-term follow-up will be needed to validate the study results. As the samples were collected before the era of widespread ultrasonographic screening for indolent thyroid cancer (Brito *et al.* 2013, Vaccarella *et al.* 2015), this cohort contained a low number of small tumors (11% of cases were smaller than 2 cm) and most of the cases presented with palpable neck mass. The inclusion of larger tumors might exaggerate the frequency of *TERT* promoter mutations in a given sample population, as the reported frequency in micro-PTC was only 4.7% (de Biase *et al.* 2015). Therefore, caution is necessary when applying the mutations as a prognostic biomarker to a different population, particularly for individuals with ultrasound-detected small micro-PTC. *TERT* mutations can coexist with *RAS* mutations as demonstrated previously (Muzza *et al.* 2015, Song *et al.* 2016) and as shown by two cases in Supplementary Table 2 (*KRAS* Q61R and *NRAS* Q61R). We could not develop more sophisticated models for FTC and PTC with wild-type *BRAF* using *RAS* mutations because of the small sample size of these subgroups with sufficient long-term follow-up.

In conclusion, the presence of *TERT* promoter mutations was found to be an independent marker of poor survival in differentiated thyroid cancer in our patient cohort. The absence of the mutations conferred negligible mortality risk during long-term follow-up in PTC patients. We predicted that the inclusion of *TERT* promoter mutation analysis with conventional clinicopathological evaluation could markedly improve the prognostication and management of individual patients with differentiated thyroid cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0219>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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