

Highly prevalent *TERT* promoter mutations in aggressive thyroid cancers

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Abstract

Mutations 1 295 228 C>T and 1 295 250 C>T (termed C228T and C250T respectively), corresponding to –124 C>T and –146 C>T from the translation start site in the promoter of the telomerase reverse transcriptase (*TERT*) gene, have recently been reported in human cancers, but not in thyroid cancers yet. We explored these mutations in thyroid cancers by genomic sequencing of a large number of primary tumor samples. We found the C228T mutation in 0 of 85 (0.0%) benign thyroid tumors, 30 of 257 (11.7%) papillary thyroid cancers (PTC), 9 of 79 (11.4%) follicular thyroid cancers (FTC), 3 of 8 (37.5%) poorly differentiated thyroid cancers (PDTTC), 23 of 54 (42.6%) anaplastic thyroid cancers (ATC), and 8 of 12 (66.7%) thyroid cancer cell lines. The C250T mutation was uncommon, but mutually exclusive with the C228T mutation, and the two mutations were collectively found in 11 of 79 (13.9%) FTC, 25 of 54 (46.3%) ATC, and 11 of 12 (91.7%) thyroid cancer cell lines. Among PTC variants, the C228T mutation was found in 4 of 13 (30.8%) tall-cell PTC (TCPTC), 23 of 187 (12.3%) conventional PTC, and 2 of 56 (3.6%) follicular variant PTC samples. No *TERT* mutation was found in 16 medullary thyroid cancer samples. The C228T mutation was associated with the *BRAF* V600E mutation in PTC, being present in 19 of 104 (18.3%) *BRAF* mutation-positive PTC vs 11 of 153 (7.2%) the *BRAF* mutation-negative PTC samples ($P=0.0094$). Conversely, *BRAF* mutation was found in 19 of 30 (63.3%) C228T mutation-positive PTC vs 85 of 227 (37.4%) C228T mutation-negative PTC samples ($P=0.0094$). We thus for the first time, to our knowledge, demonstrate *TERT* promoter mutations in thyroid cancers, that are particularly prevalent in the aggressive thyroid cancers TCPTC, PDTTC, ATC and *BRAF* mutation-positive PTC, revealing a novel genetic background for thyroid cancers.

Key Words

- ▶ *TERT* promoter mutations
- ▶ thyroid cancers
- ▶ *BRAF* V600E mutation
- ▶ telomerase reverse transcriptase
- ▶ thyroid tumorigenesis

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Introduction

Telomerase, a ribonucleoprotein complex that maintains telomere length at the end of chromosomes, plays a key role in cellular immortality and tumorigenesis (Smekalova et al. 2012, Mocellin et al. 2013). Its catalytic subunit is telomerase reverse transcriptase (TERT). Promoter mutations in the *TERT* gene on chromosome 5 have recently been reported in melanomas (Horn et al. 2013, Huang et al. 2013). Two *TERT* promoter mutations, 1 295 228 C>T and 1 295 250 C>T (termed C228T and C250T here respectively), are particularly common. They represent nucleotide changes of -124 C>T and -146 C>T (where -1 is the base just upstream of the A of the ATG translation start site) respectively in the *TERT* promoter. Both the mutations create an 11-base nucleotide stretch 5'-CCCCTTCCGGG-3', which contains a consensus binding site, GGAA (in reverse complement), for ETS transcription factors, suggesting potentially important biological relevance of these mutations. In fact, the two mutations have been demonstrated to confer increased transcriptional activity on the *TERT* promoter (Horn et al. 2013, Huang et al. 2013). These mutations are not found in normal human subjects and in the public genetic databases and are, therefore, cancer-specific somatic genetic alterations, further supporting their potentially important role in human tumorigenesis. This is consistent with the previously observed increased telomerase activities in some human cancers (Smekalova et al. 2012, Mocellin et al. 2013). Thus, *TERT* promoter mutations, by promoting the expression of the catalytic subunit of telomerase in response to ETS transcription factors, probably represent a novel mechanism by which telomerase plays an important role in human tumorigenesis. Melanomas and follicular cell-derived thyroid cancer share considerably similar genetic backgrounds; for example, they both harbor the *BRAF* V600E mutation with a high prevalence (Davies et al. 2002, Xing 2005a). We were, therefore, prompted to explore *TERT* promoter mutations in thyroid cancers in the present study.

Follicular cell-derived thyroid cancer is a common endocrine malignancy the incidence of which, similar to that of melanoma, has been rising rapidly globally in recent years (Jemal et al. 2011, Howlader et al. 2012). Follicular cell-derived thyroid cancer can be classified into several histological types (DeLellis et al. 2004), among which the most common types are papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), which account for 85–90% and 10–15% of all the thyroid cancers respectively (DeLellis et al. 2004, Jemal et al. 2011,

Howlader et al. 2012). PTC can be further classified into a few subtypes or variants, the most common of which include conventional PTC (CPTC), follicular variant PTC (FVPTC), and tall-cell PTC (TCPTC). Other subtypes of PTC, such as the columnar variant, are rare. Unlike the rare but rapidly aggressive undifferentiated anaplastic thyroid cancer (ATC; Smallridge et al. 2012), PTC and FTC are indolent differentiated thyroid cancers (DTCs). There is also poorly differentiated thyroid cancer (PDTC), which has aggressiveness between that of DTC and ATC. Parafollicular C-cell-derived medullary thyroid cancer (MTC) is uncommon. Benign thyroid tumors are far more common than thyroid cancers. Various genetic alterations have been identified in thyroid cancers, which, by aberrantly driving various signaling pathways, play a fundamental role in thyroid tumorigenesis (Xing 2013). In the present study, we examined *TERT* promoter mutations in various thyroid tumors to explore novel genetic alterations in thyroid tumorigenesis.

Subjects and methods

Thyroid tumor tissues, cell lines, and DNA

Genomic DNA was isolated from thyroid tumor tissues and cell lines using standard procedures of proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Use of human thyroid tissues was based on Institutional Review Board-approved protocols and written patient consent was obtained where appropriate. The study included 85 benign thyroid tumors, 257 PTC (consisting of 187 CPTC, 56 FVPTC, 13 TCPTC, and 1 columnar PTC), 79 conventional FTC, 8 PDTC, 54 ATC, and 16 MTC samples. Thyroid cancer cell lines included TPC1, C643, Hth7, FTC133, OCUT-1, K1, FB1, BCPAP, SW1736, KAT18, Hth74, and WRO. Their thyroid tumor origins are given in Table 1.

Identification of *TERT* promoter mutations

Standard PCR was carried out for genetic sequencing to identify *TERT* promoter mutations. Briefly, a fragment of the *TERT* promoter was amplified by PCR on genomic DNA using primers 5'-AGTGGATTCCGGGCACAGA-3' (sense) and 5'-CAGCGCTGCCTGAACTC-3' (antisense). This resulted in a PCR product of 235 bp, containing the sites where mutations C228T and C250T occur in melanomas (Horn et al. 2013, Huang et al. 2013). About

Table 1 TERT promoter mutation status of individual thyroid cancer cell lines

Cell lines	TPC1	K1	BCPAP	FTC133	WRO	C643	Hth7	OCUT-1	SW1736	KAT18	Hth74	FB1
Tumor origin	PTC	PTC	PTC	FTC	FTC	ATC	ATC	ATC	ATC	ATC	ATC	ATC
TERT promoter mutation	C228T	C228T	CC229, 228TT	C228T	Wild-type	C228T	C250T	C250T	C228T	C228T	C250T	C228T
Zygoty	Heter	Heter	Heter	Homo	Homo	Heter	Homo	Homo	Heter	Heter	Homo	Heter

PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; ATC, anaplastic thyroid cancer; Heter, heterozygous; Homo, homozygous.

40–50 ng of genomic DNA were used in the PCR, which was carried out with an initial denaturation step at 95 °C for 3 min, followed by ten cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s, and 68 °C elongation for 1 min. This was then followed by 30 cycles of the same settings except for elongation for an additional 5 s in each cycle. The PCR was completed with a final elongation step at 68 °C for 7 min. Following quality confirmation of the PCR products by gel electrophoresis, sequencing PCR was carried out using a Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and an ABI PRISM 3730 automated next generation genetic analyzer (Applied Biosystems) at the Johns Hopkins' sequencing facility. When a mutation was identified by Big Dye sequencing using the sense primer, the reaction was repeated using the antisense primer to confirm the mutation.

Identification of BRAF V600E mutation

The BRAF V600E mutation was analyzed as described previously (Hu et al. 2006). Briefly, exon 15 of the BRAF gene containing the site for the T1799A (V600E) mutation was PCR-amplified using primers TCATAATGCTTGCTCTGATAGGA (sense) and GGCCAAAATTTAATCAGTGGA (antisense), resulting in a 212 bp product. The PCR settings included one cycle of 95 °C for 5 min; two cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; two cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, followed by an extension step at 72 °C for 5 min. After quality confirmation by agarose gel electrophoresis, the PCR products were subjected to Big Dye reaction and sequencing analysis as described above for TERT mutations. All the mutations were confirmed using both the sense and antisense primers.

Results

Prevalence of TERT promoter mutations in thyroid cancer cell lines and thyroid tumors

In Fig. 1, representative electropherograms of the two TERT promoter mutations in thyroid cancer cell lines and various primary thyroid cancer tumor samples detected by both sense (Fig. 1A) and antisense (Fig. 1B) primers are shown. In Table 1, the TERT promoter mutation status of the 12 individual thyroid cancer cell lines tested is summarized. Except for the WRO cell line that harbored the wild-type TERT promoter, all the remaining 11 thyroid

cancer cell lines examined harbored *TERT* promoter mutations. PTC and FTC cell lines only harbored the C228T mutation, while the ATC cell line harbored both the C228T and C250T mutations. Table 2 summarizes *TERT* promoter mutations found in all the thyroid cancer cell lines and primary thyroid tumors. The two mutations were collectively found in 11 of the 12 (91.7%) thyroid cancer cell lines. The C228T mutation was found in 0 of 85 (0.0%) benign thyroid tumor, 30 of 257 (11.7%) PTC, 9 of 79 (11.4%) FTC, 3 of 8 (37.5%) PDTC, and 23 of 54 (42.6%) ATC samples. Among the three variants of PTC, the C228T mutation was found in 4 of 13 (30.8%) TCPTC, 23 of 187 (12.3%) CPTC, and 2 of 56 (3.6%) FVPTC samples. The single columnar PTC sample examined was positive for the C228T mutation. The C250T mutation was not found in the PTC sample, but was found in two FTC, two ATC, and three ATC cell lines. The two *TERT* promoter mutations were mutually exclusive in both thyroid cancer cell lines and thyroid cancer tumor samples and collectively found in 11 of 79 (13.9%) FTC, 25 of 54 (46.3%) ATC, and 7 of 7 (100%) ATC cell lines. No *TERT* promoter mutation was found in 16 MTC samples. Three cases had both PTC and ATC in the same thyroid gland, and in each case, both the PTC and ATC harbored the C228T mutation. Three melanoma cell lines (M14, A375, and

UACC62) examined harbored the C250T mutation (data not shown), as found in other melanoma cell lines (Horn et al. 2013, Huang et al. 2013). All the *TERT* mutations in the tumor samples were heterozygous, and some cell lines harbored a homozygous C228T or C250T mutation (Table 1). We also found a C>T mutation at position chromosome 5: 1 295 229, which is adjacent to the C228T mutation, resulting in a CC>TT tandem mutation in the BCPAP cell line (Fig. 1A). This is similar to the occasional finding of this tandem mutation in melanomas (Horn et al. 2013, Huang et al. 2013). A germline A>C (T>G on opposite strand) mutation at -57 bp from the ATG translation start site of the *TERT* gene was found in familial melanomas (Horn et al. 2013), but we did not find this mutation in any of the thyroid tumor samples or cell lines in the present study. We also did not find this mutation and other *TERT* promoter mutations in the peripheral blood DNA of 18 patients with familial PTC from a previous study (Xing 2005b).

Association of *TERT* promoter mutations with aggressive types of thyroid cancers

CPTC, FVPTC, and TCPTC account for the vast majority of PTC variants. TCPTC is classically known to be more aggressive than CPTC and FVPTC. As shown in Table 3, *TERT* promoter mutations were significantly more prevalent in the TCPTC samples than in the CPTC and FVPTC samples, 30.8% (4/13) in the former vs 10.3% (25/243) in the latter two ($P=0.046$, per two-tailed Fisher's exact test). *TERT* promoter mutations were highly significantly more prevalent in the ATC samples than in the DTC samples, 46.3% (25/54) in the former vs 12.2% (41/336) in the latter ($P=3\times 10^{-8}$). There was a trend towards a higher prevalence of *TERT* promoter mutations in the PDTC samples than in the DTC samples, 37.5% (3/8) in the former vs 12.2% (41/336) in the latter ($P=0.069$). Statistical significance was not reached, probably due to the relatively small number of PDTC samples.

Association of *TERT* promoter mutation C228T with *BRAF* V600E mutation in PTC

BRAF V600E mutation, which activates the MAPK pathway, is the most common mutation in thyroid cancers, particularly in PTC (Xing 2005a). We, therefore, analyzed the relationship between this mutation and *TERT* promoter mutation C228T in PTC. As shown in Table 4, *TERT* promoter mutation C228T more commonly occurred in the PTC samples harboring the *BRAF* V600E

Table 2 *TERT* promoter mutations in thyroid tumors

Samples	Mutation C228T (n/N (%))	Mutation C250T (n/N (%))	Collective mutations (n/N (%))
Thyroid cancer cell lines			
PTC	3/3 (100.0)	0/3 (0.0)	3/3 (100.0)
FTC	1/2 (50.0)	0/2 (0.0)	1/2 (50.0)
ATC	4/7 (57.1)	3/7 (42.9)	7/7 (100.0)
All	8/12 (66.7)	3/12 (25.0)	11/12 (91.7)
Thyroid tumors			
Benign tumor	0/85 (0.0)	0/85 (0.0)	0/85 (0.0)
PTC			
CPTC	23/187 (12.3)	0/187 (0.0)	23/187 (12.3)
FVPTC	2/56 (3.6)	0/56 (0.0)	2/56 (3.6)
TCPTC	4/13 (30.8)	0/13 (0.0)	4/13 (30.8)
Columnar	1/1 (100.0)	0/1 (0.0)	1/1 (100.0)
All	30/257 (11.7)	0/257 (0.0)	30/257 (11.7)
FTC	9/79 (11.4)	2/79 (2.5)	11/79 (13.9)
DTC	39/336 (11.6)	2/336 (0.6)	41/336 (12.2)
PDTC	3/8 (37.5)	0/8 (0.0)	3/8 (37.5)
ATC	23/54 (42.6)	2/54 (3.7)	25/54 (46.3)
MTC	0/16 (0.0)	0/16 (0.0)	0/16 (0.0)

PTC, papillary thyroid cancer; CPTC, conventional PTC; FVPTC, follicular variant PTC; TCPTC, tall-cell PTC; FTC, follicular thyroid cancer; DTC, differentiated thyroid cancer (combination of PTC and FTC); PDTC, poorly DTC; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer.

Table 3 Association of *TERT* promoter mutations with aggressive thyroid cancers

Types of thyroid cancers	Collective <i>TERT</i> promoter mutations (n/N (%))	P value ^a
TCPTC	4/13 (30.8)	0.046
CPTC+ FVPTC	25/243 (10.3)	
ATC	25/54 (46.3)	3×10^{-8}
DTC	41/336 (12.2)	
PDTC	3/8 (37.5)	0.069
DTC	41/336 (12.2)	

PTC, papillary thyroid cancer; TCPTC, tall-cell PTC; CPTC, conventional PTC; FVPTC, follicular variant PTC; DTC, differentiated thyroid cancer (combination of PTC and FTC); PDTC, poorly DTC; ATC, anaplastic thyroid cancer.
^aPer two-tailed Fisher's exact test.

mutation than in the PTC samples harboring the wild-type *BRAF* gene, with a prevalence of 18.3% (19/104) in the former vs 7.2% (11/153) in the latter ($P=0.0094$, per two-tailed Fisher's exact test). Conversely, *BRAF* mutation more commonly occurred in the PTC samples harboring the *TERT* promoter mutation than in the PTC samples harboring the wild-type *TERT*, 63.3% (19/30) in the former vs 37.4% (85/227) in the latter ($P=0.0094$). Thus, the majority of the *TERT* promoter mutation-positive PTC samples harbored the *BRAF* V600E mutation. Several cases of ATC had both *BRAF* V600E and *TERT* mutations, but the relationship of the two types of mutations could not be statistically analyzed in this cancer due to the small number of *BRAF* mutation-positive cases (Table 4).

Discussion

The recent discovery of *TERT* promoter mutations in melanomas is the first example, to our knowledge, indicating that mutations in gene promoters may also play an important oncogenic role (Horn et al. 2013, Huang et al. 2013). This represents a novel genetic mechanism in human tumorigenesis. A subsequent report of the existence of *TERT* promoter mutations in other human cancers (Killela et al. 2013) and our report on the high prevalence of

these mutations in bladder cancer and glioblastoma (Liu et al. 2013) suggest that *TERT* promoter mutations may play a huge role in human tumorigenesis. We report here for the first time, to our knowledge, that common *TERT* promoter mutations are also in observed thyroid cancer.

We found no *TERT* promoter mutations in para-follicular C-cell-derived MTC samples, consistent with similar findings in a recent study on 24 MTC samples (Killela et al. 2013). However, due to the relatively small number of samples examined, the status of *TERT* promoter mutations in MTC cannot be definitively concluded. In contrast, in the analysis of a large cohort of follicular cell-derived thyroid cancer samples in the present study, we found a common occurrence of *TERT* promoter mutations in both PTC and FTC samples, suggesting a role of these mutations in the tumorigenesis of a subgroup of these DTCs. The lack of *TERT* promoter mutations in benign thyroid tumor samples suggests that these mutations are malignancy-specific and may be relatively late genetic events along the line of thyroid tumorigenesis. Consistent with this idea is the strikingly higher prevalence of *TERT* promoter mutations in PDTC and ATC than in DTCs; PDTC and ATC have partially and completely lost differentiation respectively and are the most aggressive thyroid cancers. This raises the possibility that *TERT* promoter mutations may play a particular role in the de-differentiation of DTCs and hence their conversion to poorly or undifferentiated aggressive thyroid cancers. This possibility is consistent with the finding in three cases in which co-existing PTC and ATC in the same thyroid gland harbored *TERT* promoter mutation C228T. The prevalence of *TERT* promoter mutations was extremely high in thyroid cancer cell lines (91.7%), which is in contrast to the low prevalence of 16% (24/150) in general cancer cell lines from the Cancer Cell Line Encyclopedia (Huang et al. 2013), but is similar to the high prevalence of 74% (125/168) in melanoma cell lines (Horn et al. 2013). This result is again consistent with the idea that *TERT* promoter mutations may play a role in the de-differentiation of thyroid cancer cells since thyroid cancer cell lines in

Table 4 Association of *TERT* promoter C228T mutation with *BRAF* V600E mutation in PTC

Tumor type	<i>TERT</i> C228T mutation (n/N (%))		P value ^a	<i>BRAF</i> V600E mutation (n/N (%))		P value ^a
	BRAF–	BRAF+		TERT–	TERT+	
PTC	11/153 (7.2)	19/104 (18.3)	0.0094	85/227 (37.4)	19/30 (63.3)	0.0094
ATC	20/44 (45.5)	5/10 (50.0)	1.0	5/29 (17.2)	5/25 (20.0)	1.0

PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer.
^aPer two-tailed Fisher's exact test.

culture commonly become de-differentiated (van Staveren *et al.* 2007). Interestingly, among the three common variants of PTC, TCPTC harbored *TERT* promoter mutations with the highest prevalence. TCPTC is a relatively uncommon but more aggressive PTC variant than CPTC and FVPTC (Xing *et al.* 2005, Ghossein *et al.* 2007, LiVolsi 2010). It is possible that *TERT* promoter mutations play a role in the aggressiveness of this unique PTC variant. This is again consistent with the idea that *TERT* promoter mutations may play a role in the development of progression and aggressiveness of thyroid cancers.

As in many other human cancers in which telomerase activities are increased (Smekalova *et al.* 2012, Mocellin *et al.* 2013), increased telomerase activities have also been found in thyroid cancers but not in normal thyroid tissues or benign thyroid tumors, suggesting a role of this enzyme in thyroid cancer tumorigenesis (Capezzone *et al.* 2009). Both *TERT* promoter C228T and C250T mutations create binding sites for ETS transcription factors, which subsequently promote the expression of *TERT* (Horn *et al.* 2013, Huang *et al.* 2013). Thus, *TERT* promoter mutations may contribute to thyroid tumorigenesis by aberrantly promoting the expression of *TERT*. Interestingly, some ETS factors are targets of the MAPK signaling pathway (Janknecht *et al.* 1995, Whitmarsh *et al.* 1995, Strahl *et al.* 1996). The MAPK pathway aberrantly activated by BRAF V600E plays a fundamental role in the tumorigenesis and progression of PTC (Xing 2013). It is thus possible that *TERT* promoter mutations may join the mechanisms involving the MAPK signaling in thyroid tumorigenesis. Consistent with this hypothesis is the particularly high prevalence of *TERT* promoter mutations in BRAF V600E mutation-positive PTC and *vice versa* found in the present study. The preferential occurrence of *TERT* promoter mutations in BRAF V600E mutation-positive PTC is also consistent with the hypothesis discussed above that these *TERT* promoter mutations may play a role in the aggressiveness of thyroid cancers since BRAF V600E mutation-positive PTC is more aggressive than PTC with wild-type BRAF (Xing *et al.* 2005, 2013a). The association between *TERT* promoter and BRAF V600E mutations creates a unique mechanism for the amplification of *TERT* expression, in which *TERT* promoter mutations create binding sites for ETS transcription factors, which, upon activation by BRAF V600E-promoted MAPK signaling, initiate or augment the expression of *TERT*. Thus, the co-existence of *TERT* promoter and BRAF V600E mutations conceivably confers thyroid cancers with a unique survival advantage. New treatments targeting

molecular targets, such as BRAF V600E, are being actively sought and tested for thyroid cancers (Xing *et al.* 2013b). The finding of *TERT* promoter mutations in thyroid cancers opens an exciting possibility for the development of novel therapeutic agents targeting *TERT* in thyroid cancer patients. Given the association of *TERT* promoter mutations with BRAF V600E mutation and their presumed interaction through enhancement of the function of ETS transcription factors in regulating the expression of *TERT*, this therapeutic strategy may be particularly effective in patients with both *TERT* promoter mutations and BRAF V600E mutation.

In summary, herein, we report for the first time, to our knowledge, common *TERT* promoter mutations in thyroid cancers, which are particularly prevalent in aggressive types of thyroid cancers and in BRAF V600E mutation-positive PTC. Their occurrence patterns in various types of thyroid cancers suggest that these *TERT* promoter mutations may play a role in the de-differentiation, progression, and aggressiveness of thyroid cancers. The discovery of this novel genetic background of thyroid cancers opens exciting new opportunities for biological and clinical research of thyroid cancers.

Declaration of interest

M Xing received royalties as a co-holder of a licensed USA patent related to the discovery and clinical characterization of BRAF V600E mutation in thyroid cancers.

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