TERT promoter mutations are associated with distant metastases in papillary thyroid carcinoma

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Abstract

Objective: Transcriptional activating mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene were reported at high frequency in aggressive poorly differentiated and anaplastic thyroid cancers. By contrast, the relevance of these mutations in the metastatic behavior of well-differentiated thyroid cancer is still to be defined. The aim of this work was to investigate the frequency of *TERT* promoter mutations in a remarkable cohort of well-differentiated papillary thyroid carcinoma that developed distant metastases (DM-PTCs) and to establish whether these mutations may be predictive of metastatic behavior.

Design: We analyzed the frequency of *TERT* promoter mutations in a group of 43 highly aggressive DM-PTCs. As controls, we analyzed these mutations in a group of 78 PTCs without distant metastases (control-PTCs). The possible correlation between *TERT* promoter mutations and BRAF V600E mutation was also investigated.

Methods: *TERT* promoter mutational status was evaluated by direct sequencing of the hotspot harboring the C228T and the C250T mutations.

Results: In the overall cohort of 121 PTCs analyzed, 17% of cases (21/121) carried a mutation in the *TERT* promoter. Noticeably, 33% of DM-PTCs were mutated in the *TERT* promoter while only 9% of the control-PTCs showed a mutation in this locus. We also observed a positive association between BRAF V600E and *TERT* C228T mutations in the cohort of DM-PTCs.

Conclusions: These results indicate that *TERT* promoter mutations are associated with the development of distant metastases in PTCs and may help in predicting aggressive behavior in this type of tumor.

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Introduction

Thyroid tumors are the most common malignancies of the endocrine system and their incidence increased significantly over the past two decades worldwide (1, 2, 3). Based on histological and cytological features, follicular-cell derived tumors are classified into three categories: well-differentiated, poorly differentiated, and anaplastic. Aggressiveness and lethality of these tumors are inversely correlated with the degree of their differentiation (4). Well-differentiated papillary thyroid carcinomas (PTC), the most common type of thyroid cancer, are usually considered indolent lesions with slow rate of growth, low

metastatic potential, and favorable outcome (up to 95% of survival after 10 years) (3, 5). Nevertheless, the occurrence of aggressively behaving well-differentiated carcinomas is not a rare event and there is an urgent need of more accurate prognostic tools to predict the spread of these lesions to distant sites in the early phases of diagnosis. Besides age, the presence of distant metastases, occurring in a small percentage (~10%) of the entire PTC population (5, 6, 7), represents the major clinical feature reliably associated with a negative outcome of PTC patients. During progression from local to metastatic

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Table 1 Clinical and pathological features of well-differentiated papillary thyroid carcinomas with distant metastases (DM-PTCs) or without distant metastases (control-PTCs) used in this study.

Clinico-pathological features	DM-PTCs	Control-PTCs	P value
Total	43	78	
Mean follow-up, months (range)	102.1 ± 76.8 (2–391)	136.3 ± 59.2 (15–369)	0.01
Mean age at diagnosis, years (range)	54.8 ± 19.1 (19–90)	44.4 ± 16.8 (16–78)	0.003
Sex			
Females	28 (0.65)	55 (0.71)	0.54
Males	15 (0.35)	23 (0.29)	
Histological diagnosis			
CPTCs	20 (0.46)	45 (0.58)	0.39
TCV-PTCs	15 (0.35)	20 (0.26)	
FV-PTCs	6 (0.14)	9 (0.12)	
SD-PTCs	0	2 (0.03)	
ST-PTCs	2 (0.05)	1 (0.01)	
Pathologic stage at presentation			
I	2 (0.05)	47 (0.60)	<0.001
II	9 (0.21)	4 (0.05)	
III	7 (0.16)	18 (0.23)	
IV	25 (0.58)	9 (0.12)	
Vascular invasion ^a			
Present	29 (0.76)	24 (0.40)	<0.001
Absent	9 (0.24)	36 (0.60)	
NA	5	18	
Follow-up status			
NED	5 (0.12)	66 (0.85)	<0.001
AWD ^A	16 (0.37)	2 (0.02)	
DOD	22 (0.51)	0	
DOC	0	10 (0.13)	

CPTC, classic PTC; TCV-PTC, tall cell-variant PTC; FV-PTC, follicular-variant PTC; SD-PTC, sclerosing diffuse PTC; ST-PTC, solid trabecular PTC; NED, alive with no evidence of disease; AWD, alive with disease (^Aalive with metastatic disease in DM-PTCs or alive with neck recurrence in control-PTCs); DOD, dead of disease; DOC, dead of other causes.

^aVascular invasion was defined as the presence of intravascular tumor cells either covered by endothelium or associated with thrombus, affecting lymphatic or blood vessels, and was analyzed in 38 DM-PTCs and in 60 control-PTCs.

disease, tumor cells change their genomic profile to acquire adaptive aggressive features. Owing to the low frequency of distantly metastatic PTCs, very little is currently known on the genetic alterations that occur during the spreading to distant sites of these tumors.

Unlimited replicative potential is a major hallmark of cancer cells (8). As a consequence of the high rate of proliferation, the telomere repeats length tends to shorten very rapidly in cancer cells. Telomerase is a large protein complex which protects the telomere repeats from erosion, thus antagonizing replicative senescence and apoptosis (9, 10). Indeed, abnormal function of telomerase has been described as a common feature in several types of cancer (11). Telomerase activity is mainly dependent on the expression of its rate-limiting catalytic subunit, namely the telomerase reverse transcriptase (TERT). Recently, mutations in the promoter of *TERT* gene were described to occur with high frequency in different types of solid tumors (12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22). In particular, *TERT* promoter mutations were mainly found

in two hot spots in positions –124 bp (chr5: 1 295 228 G>A, hereafter termed C228T) and –146 bp (chr5: 1 295 250 G>A, hereafter termed C250T) upstream the gene transcription starting site. These alterations were

Table 2 Occurrence and frequencies of *TERT* promoter mutations in well-differentiated papillary thyroid carcinomas with distant metastases (DM-PTCs) or without distant metastases (control-PTCs).

	Total PTCs	DM-PTCs	Control-PTCs
<i>n</i>	121	43	78
<i>n</i> PTCs mutated in <i>TERT</i> promoter (frequency)	21 (0.17)	14 (0.33) ^a	7 (0.09) ^a
Type of mutation			
C228T	12 (0.10)	11 (0.26)	1 (0.013)
C250T	5 (0.04)	3 (0.07)	2 (0.025)
CC228-229TT	2 (0.02)	0	2 (0.025)
C229T	1 (<0.01)	0	1 (0.013)
C232T	1 (<0.01)	0	1 (0.013)

P value=0.002.

^aFisher's exact test between *TERT* mutation frequencies in DM-PTCs and control-PTCs.

found to increase promoter activity and *TERT* expression, most likely introducing new consensus sites for transcription factors belonging to the ETS family.

TERT promoter mutations were also described in thyroid cancer, where C228T represents the most common mutation. The frequency of *TERT* promoter mutations is quite low in well-differentiated thyroid cancer (7.5–25% depending on series), but it increases significantly from well to poorly differentiated and undifferentiated carcinomas, up to 50% of all cases, indicating a strong association of these mutations with the aggressiveness and the metastatic spreading of thyroid malignancy (23, 24, 25, 26, 27, 28).

The aim of this work was to assess the occurrence and the frequency of *TERT* promoter mutations in a large cohort of PTCs that developed distant metastases and to establish whether the presence of these mutations may hold promise of useful prognostic biomarker in this type of cancer.

Subjects and methods

Patient selection

For this study, 43 cases of aggressive well-differentiated PTCs that developed distant metastases outside the neck (mainly in lungs, brain, and bones; DM-PTCs) were retrieved from the Thyroid Tumor archive of the Pathology Unit of the Arcispedale S. Maria Nuova, which comprises 2200 cases of thyroid cancers diagnosed from 1979 to 2013. From the same archive, 78 control PTCs without distant metastases were retrieved. Of those, 47 PTCs developed neck lymph node metastases (LNMs), while 31 did not develop LNMs and were confined to the thyroid gland.

Tumor cases were screened by two authors (S Piana and M Ragazzi) and classified according to the criteria recommended by the World Health Organization Classification of Tumors (29), in order to exclude high-grade carcinomas, and staged according to AJCC Cancer Staging Manual, 7th edition (30).

Control samples were not consecutive cases and were selected according to the metastases status (N0 or N1 and M0) and to the clinical data and tissue availability. It was not possible to obtain matched pair for age, due to the average older age of DM-PTCs patients.

In addition, 16 LNMs derived from 15 of the primary PTCs cases described above were retrieved from the archive. These included ten LNMs from nine primary

DM-PTCs (two LNMs derived from a single primary DM-PTCs) and six LNMs from six primary control-PTCs.

Part of these samples corresponded to the cases described in previous studies (31, 32). All the patients underwent total thyroidectomy and in 80% of the cases they underwent also ipsilateral central neck dissection. Average follow-up time for all cases was 124.1 ± 67.7 months. Clinical and pathological features of DM-PTCs and control cases are summarized in Table 1. This project has been approved by the Local Ethics Committee.

Tissues selection and genomic DNA extraction

Genomic DNA was extracted from five 5 μ m-thick slices of formalin-fixed and paraffin-embedded PTCs and LNMs samples, by using the Biostic FFPE Tissue DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). The tumor samples were manually dissected under microscopic guidance by the pathologists M Ragazzi and S Piana and any nontumoral tissue surrounding the tumor was pared away.

TERT promoter mutation analysis

The *TERT* promoter mutations were analyzed by PCR amplification and direct Sanger sequencing of the hot spots was previously described. Briefly, a 231-bp region of the *TERT* promoter containing the position of C228T and

Figure 1

TERT promoter mutation status in matched primary PTCs (squares) and lymph node metastases (LNMs, circles). Gray shapes are *TERT* C228T mutated and white shades are WT.

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Table 3 Association between *TERT* promoter mutation and clinical and pathological features in well-differentiated PTCs with distant metastases (DM-PTCs) and in PTCs without distant metastases (control-PTCs).

	<i>TERT</i> WT	<i>TERT</i> mutated	<i>P</i> value
Total PTCs (n=121)	<i>n</i> =100	<i>n</i> =21	
Age at diagnosis (years)	45.6±17.6	59.8±17.1	0.001
Sex			0.8
Females	69 (0.69)	14 (0.66)	
Males	31 (0.31)	7 (0.33)	
Histological diagnosis			0.03
CPTCs	60 (0.60)	6 (0.29)	
TCV-PTCs	24 (0.24)	11 (0.52)	
FV-PTCs	13 (0.13)	2 (0.09)	
SD-PTCs	1 (0.01)	1 (0.05)	
ST-PTCs	2 (0.02)	1 (0.05)	
Pathologic stage at presentation			0.02
I	46 (0.46)	3 (0.14)	
II	11 (0.11)	2 (0.10)	
III	20 (0.20)	5 (0.24)	
IV	23 (0.23)	11 (0.52)	
Vascular invasion (n=98)			0.9
Present	42 (0.53)	11 (0.58)	
Follow-up status			0.001
NED	66 (0.66)	5 (0.24)	
AWD ^A	14 (0.14)	4 (0.19)	
DOD	13 (0.13)	9 (0.43)	
DOC	7 (0.07)	3 (0.14)	
Distant metastases			0.002
Present	29 (0.29)	14 (0.67)	
LNMs			0.27
Present	72 (0.72)	18 (0.85)	
DM-PTCs (n=43)	<i>n</i> =29	<i>n</i> =14	
Age at diagnosis (years)	50±19.2	64.9±14.7	0.02
Sex			1
Females	19 (0.66)	9 (0.64)	
Males	10 (0.34)	5 (0.36)	
Histological diagnosis			0.06
CPTCs	17 (0.59)	3 (0.22)	
TCV-PTCs	7 (0.24)	8 (0.57)	
FV-PTCs	4 (0.14)	2 (0.14)	
SD-PTCs	0	0	
ST-PTCs	1 (0.03)	1 (0.07)	
Pathologic stage at presentation			0.32
I	2 (0.07)	0 (0.0)	
II	8 (0.27)	1 (0.07)	
III	4 (0.14)	3 (0.21)	
IV	15 (0.52)	10 (0.72)	
Vascular invasion (n=38)			0.73
Present	20 (0.80)	9 (0.69)	
Follow-up status			0.57
NED	4 (0.14)	1 (0.07)	
AWD ^A	12 (0.41)	4 (0.29)	
DOD	13 (0.45)	9 (0.64)	
DOC	0	0	
Control-PTCs (n=78)	<i>n</i> =71	<i>n</i> =7	
Age at diagnosis (years)	43.8±16.7	49.9±18.4	0.38
Sex			1
Females	50 (0.70)	5 (0.71)	
Males	21 (0.30)	2 (0.29)	
Histological diagnosis			0.2

Table 3 Continued.

	<i>TERT</i> WT	<i>TERT</i> mutated	<i>P</i> value
CPTCs	43 (0.60)	3 (0.43)	
TCV-PTCs	17 (0.24)	3 (0.43)	
FV-PTCs	9 (0.13)	0	
SD-PTCs	1 (0.015)	1 (0.14)	
ST-PTCs	1 (0.015)	0	
Pathologic stage at presentation			0.61
I	44 (0.62)	3 (0.43)	
II	3 (0.04)	1 (0.14)	
III	16 (0.23)	2 (0.29)	
IV	8 (0.11)	1 (0.14)	
Vascular invasion (n=60)			1
Present	22 (0.41)	2 (0.33)	
Follow-up status			0.04
NED	62 (0.87)	4 (0.57)	
AWD ^A	2 (0.03)	0	
DOD	0	0	
DOC	7 (0.10)	3 (0.43)	
LNMs			
Present	43 (0.61)	4 (0.57)	1

CPTC, classic PTC; TCV-PTC, tall cell-variant PTC; FV-PTC, follicular-variant PTC; SD-PTC, sclerosing diffuse PTC; ST-PTC, solid trabecular PTC; NED, alive with no evidence of disease; AWD, alive with disease (^Aalive with metastatic disease in DM-PTCs or alive with neck recurrence in control-PTCs); DOD, dead of disease; DOC, dead of other causes.

C250T mutations was PCR-amplified using 40–50 ng of genomic DNA: the forward primer 5'-GGATTCGCGGG-CACAGAC-3' and the reverse primer 5'-AGCGCTGCCT-GAAACTCG-3'. The nested-PCR protocol consisted of two steps, each comprising an initial denaturation of 3 min at 94 °C and 35 amplification cycles (denaturation for 30 s at 94 °C, annealing for 30 s at 60 and 65 °C, and extension for 30 s at 72 °C). Purified PCR products were subjected to standard dye-terminator sequencing using the Genome Laboratory DTCS Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA, USA) and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.).

Statistical analysis

Statistical analyses were performed by R Statistical Software Package (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Data are reported as frequencies or as mean±s.d., and *TERT* promoter mutations frequencies were compared between groups by Fisher's exact test.

The Mann-Whitney-Wilcoxon test (for continuous variables), the χ^2 test (for categorical variables with more than two categories), and Fisher's exact test (for categorical variables with two categories) were used to compare the clinicopathological parameters between *TERT*-mutated

and *TERT*-WT PTCs. The same tests were used for the comparison between PTCs without *BRAF* or *TERT* mutations and the following three groups: PTCs with only *TERT* mutations, PTCs with only *BRAF* mutation, and PTCs with *TERT* and *BRAF* mutations coexistence).

Survival curves were plotted by the Kaplan–Meier method with the log-rank statistics. In the survival analysis, only deaths attributable to the thyroid carcinoma (disease-specific mortality) were considered. $P < 0.05$ values were considered as threshold for significance.

Results

TERT promoter is frequently mutated in aggressive PTCs with distant metastases

The mutational status of a hotspot region within *TERT* promoter was analyzed in a cohort of 121 PTC, including 43 aggressive PTCs with distant metastases outside the neck and 78 control-PTCs.

The DM-PTC group showed older mean age at diagnoses, higher pathological stage at presentation, higher incidence of vascular invasion, and worse status at follow up compared with the control group, while the two groups did not differ for gender and histotype frequencies (Table 1).

Table 2 gives the results of the mutation analysis. *TERT* promoter mutations were found in 21 out of 121 PTCs, corresponding to 17% of the overall cohort. We identified four different *TERT* promoter mutations, occurring in a mutually exclusive manner. Mutation C228T was the most frequent alteration (found in 10% of all cases) followed by the C250T mutation (4% of all cases). Double mutation CC228–229TT was found in two cases and the mutations C229T and C232T were found in one sample each. This latter has never been previously reported. The relative frequency among the detected mutations is in line with the available data in thyroid cancer (23, 24).

We observed a strikingly higher frequency of *TERT* promoter mutations in the group of DM-PTCs compared with controls. Indeed, 14 out of 43 DM-PTCs, corresponding to 33% of cases, harbored *TERT* promoter mutations. By contrast, only seven out of 78 control-PTCs were *TERT*-mutated, corresponding to 9% of cases. This difference was statistically significant (Fisher's exact test; $P = 0.002$). In the DM-PTCs group, only the C228T and C250T mutations were found, with C228T by far being the most frequent alteration (corresponding to 78.6% of detected mutations). In the control-PTCs group the different mutations were evenly distributed.

TERT promoter mutation is not associated with the development of LNM

Within the control-PTCs group, 47 cases developed neck LNMs while 31 were confined to the thyroid. We did not observe any difference in the occurrence of *TERT* mutations between these two subgroups. Indeed, four out of the 47 PTCs with LNMs displayed *TERT* promoter mutations (frequency: 0.08), while three *TERT*-mutated PTCs were detected in the group of 31 PTCs without LNMs (frequency: 0.10; $P = 0.99$). In order to evaluate the correspondence of *TERT* mutational status between primary and matched LNMs, we analyzed 16 LNM samples originated from 15 primary PTCs, including ten WT and five *TERT*-mutated cases (Fig. 1). We observed correspondence in the *TERT* genotype in all but two LNM samples, in which the mutation was respectively lost or acquired at the metastatic site. Intriguingly, one of these cases was a WT DM-PTCs, which developed two subsequent LNMs before the distant spreading of the disease. Of these two LNMs, the earlier was *TERT*-WT while the later had acquired the C228T mutation. This observation seems to suggest that, even if not commonly, *TERT* promoter mutations can be acquired in late phases of tumor progression.

TERT promoter mutations are associated with aggressive clinico-pathological features of PTCs

Table 3 shows the results of the association analysis between *TERT* promoter mutations and different clinico-pathological features of PTCs. In the overall cohort of 121 samples, *TERT* promoter mutations were significantly associated with older age, tall cell-variant (TCV) histotype, advanced stage, DM occurrence, and worse outcome.

Table 4 Association between the occurrences of *TERT* promoter mutations and *BRAF* V600E mutation in well-differentiated PTCs.

	BRAF+	BRAF–	P value
Total PTCs			
<i>TERT</i> C228T	9/54 (0.16)	5/67 (0.07)	NS
<i>TERT</i> C250T	2/54 (0.04)	3/67 (0.04)	NS
<i>TERT</i> all mutations	12/54 (0.22)	9/67 (0.13)	NS
DM-PTCs			
<i>TERT</i> C228T	7/15 (0.46)	4/28 (0.14)	0.03
<i>TERT</i> C250T	0/15 (0.0)	3/28 (0.11)	–
<i>TERT</i> all mutations	7/15 (0.46)	7/28 (0.25)	NS
Control-PTCs			
<i>TERT</i> C228T	2/39 (0.05)	1/39 (0.03)	NS
<i>TERT</i> C250T	2/39 (0.05)	0/39 (0.0)	–
<i>TERT</i> all mutations	5/39 (0.13)	2/39 (0.05)	NS

Table 5 Association of *TERT* promoter mutations, BRAF V600E mutation, and their coexistence with clinical and pathological features in the total cohort of well-differentiated PTCs, in PTCs with distant metastases (DM-PTCs), and in PTCs without distant metastases (control-PTCs).

	No mutation	Only <i>TERT</i> mutation	<i>P</i>	Only <i>BRAF</i> mutation	<i>P</i>	<i>TERT</i> + <i>BRAF</i> mutation	<i>P</i>
Total PTCs (n=121)	n=58	n=9		n=42		n=12	
Age at diagnosis (years)	42.8±17.2	53.7±20.9	0.09	49.5±17.5	0.06	64.5±12.7	0.0004
Sex			0.88		1		0.70
Females	40 (0.69)	7 (0.78)		29 (0.69)		7 (0.58)	
Males	18 (0.31)	2 (0.22)		13 (0.31)		5 (0.41)	
Histological diagnosis			0.28		0.03		0.0003
CPTCs	37 (0.64)	3 (0.33)		23 (0.55)		3 (0.25)	
TCV-PTCs	8 (0.14)	2 (0.22)		16 (0.38)		9 (0.75)	
FV-PTCs	10 (0.17)	2 (0.22)		3 (0.07)		0	
SD-PTCs	1 (0.02)	1 (0.11)		0		0	
ST-PTCs	2 (0.03)	1 (0.11)		0		0	
Pathologic stage at presentation			0.43		0.15		0.03
I	27 (0.47)	2 (0.22)		19 (0.45)		1 (0.08)	
II	9 (0.15)	1 (0.11)		2 (0.05)		1 (0.08)	
III	8 (0.14)	2 (0.22)		12 (0.29)		3 (0.25)	
IV	14 (0.24)	4 (0.44)		9 (0.21)		7 (0.59)	
Vascular invasion (n=98)			0.45		0.26		0.31
Present	19 (0.59)	7 (0.78)		15 (0.45)		4 (0.40)	
Follow-up status			0.12		0.16		0.002
NED	37 (0.64)	3 (0.33)		29 (0.69)		2 (0.17)	
AWD ^A	11 (0.19)	2 (0.22)		3 (0.07)		2 (0.17)	
DOD	8 (0.14)	4 (0.44)		5 (0.12)		5 (0.41)	
DOC	2 (0.03)	0		5 (0.12)		3 (0.25)	
Distant metastases			0.02		0.07		0.20
Present	21 (0.36)	7 (0.78)		8 (0.19)		7 (0.58)	
LNMs			0.10		1		1
Present	42 (0.72)	9 (1)		30 (0.71)		9 (0.75)	
DM-PTCs (n=43)	n=21	n=7		n=8		n=7	
Age at diagnosis (years)	47.7±16.8	61.4±16.1	0.04	55.7±24.9	0.28	68.2±13.2	0.01
Sex			0.66		0.20		1
Females	12 (0.57)	5 (0.71)		7 (0.87)		4 (0.57)	
Males	9 (0.43)	2 (0.29)		1 (0.13)		3 (0.43)	
Histological diagnosis			1		1		0.31
CPTCs	14 (0.67)	2 (0.29)		3 (0.38)		1 (0.14)	
TCV-PTCs	2 (0.09)	2 (0.29)		5 (0.62)		6 (0.86)	
FV-PTCs	4 (0.19)	2 (0.29)		0		0	
ST-PTCs	1 (0.05)	1 (0.13)		0		0	
Pathologic stage at presentation			0.55		0.58		0.37
I	1 (0.05)	0		1 (0.13)		0	
II	6 (0.29)	1 (0.14)		2 (0.25)		0	
III	2 (0.09)	2 (0.29)		2 (0.25)		1 (0.14)	
IV	12 (0.57)	4 (0.57)		3 (0.37)		6 (0.86)	
Vascular invasion (n=38)			1		0.6		0.13
Present	15 (0.83)	6 (0.86)		5 (0.71)		3 (0.50)	
Follow-up status			0.64		0.47		0.26
NED	3 (0.14)	1 (0.14)		1 (0.12)		0	
AWD ^A	10 (0.48)	2 (0.29)		2 (0.25)		2 (0.29)	
DOD	8 (0.38)	4 (0.57)		5 (0.63)		5 (0.71)	
Control-PTCs (n=78)	n=37	n=2		n=34		n=5	
Age at diagnosis (years)	40.0±17.0	26.5±6.3	0.29	48.0±15.5	0.03	59.2±10.2	0.02
Sex			1		0.43		0.59
Females	28 (0.76)	2 (1)		22 (0.65)		3 (0.60)	
Males	9 (0.24)	0		12 (0.35)		2 (0.40)	
Histological diagnosis			0.06		0.33		0.25
CPTCs	23 (0.62)	1 (0.50)		20 (0.59)		2 (0.40)	
TCV-PTCs	6 (0.16)	0		11 (0.32)		3 (0.60)	
FV-PTCs	6 (0.16)	0		3 (0.09)		0	

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Table 5 Continued.

	No mutation	Only <i>TERT</i> mutation	<i>P</i>	Only <i>BRAF</i> mutation	<i>P</i>	<i>TERT</i> + <i>BRAF</i> mutation	<i>P</i>
SD-PTCs	1 (0.03)	1 (0.50)		0		0	
ST-PTCs	1 (0.03)	0		0		0	
Pathologic stage at presentation			0.84		0.06		0.17
I	26 (0.70)	2 (1)		18 (0.53)		1 (0.20)	
II	3 (0.08)	0		0		1 (0.20)	
III	6 (0.16)	0		10 (0.29)		2 (0.40)	
IV	2 (0.06)	0		6 (0.18)		1 (0.20)	
Vascular invasion (<i>n</i> =60)			1		0.78		0.63
Present	12 (0.43)	1 (0.50)		10 (0.38)		1 (0.25)	
Follow-up status			0.91		0.41		0.001
NED	34 (0.92)	2 (1)		28 (0.82)		2 (0.40)	
AWD ^A	1 (0.03)	0		1 (0.03)		0	
DOC	2 (0.05)	0		5 (0.15)		3 (0.60)	
LNMs			0.50		0.63		0.64
Present	21 (0.57)	2 (1)		22 (0.65)		2 (0.4)	

P values refer to the comparison of the no mutation group with the group in the column at the left of the *P* value. CPTC, classic PTC; TCv-PTC, tall cell-variant PTC; FV-PTC, follicular-variant PTC; SD-PTC, sclerosing diffuse PTC; ST-PTC, solid trabecular PTC; NED, alive with no evidence of disease; AWD, alive with disease (^Aalive with metastatic disease in DM-PTCs or alive with neck recurrence in control-PTCs); DOD, dead of disease; DOC, dead of other causes.

As this cohort of samples was selected in order to have a representative number of aggressive PTCs with distant metastases, it is likely that this association may reflect the higher frequency of this mutation within the DM-PTC group. Indeed, when we performed this analysis in DM-PTCs and control-PTCs groups independently, only age at diagnosis remained significantly associated with the presence of *TERT* mutations in the DM-PTCs group, while no significant association was observed in the control group.

Association of *TERT* promoter mutations, *BRAF* V600E mutation, and their coexistence with clinico-pathological features of PTCs

The *BRAF* V600E mutation is the most common genetic alteration in PTCs, with a frequency of about 50% among all cases. Recently, a possible association between this mutation and the occurrence of *TERT* promoter mutations has been described and a biological cooperation between these alterations has been suggested (33). Thus, we investigated the association between the occurrence of *TERT* promoter mutations and *BRAF* V600E (Table 4). *BRAF* V600E mutation was present in 54 of the 121 PTC samples analyzed (frequency: 0.44). As previously described, no association between the occurrence of this mutation and the presence of distant metastases was detected (31, 32). An association between the presence of *TERT* C228T and *BRAF* V600E was observed in the DM-PTCs group (46% of *TERT* C228T-mutated in *BRAF*-positive cases vs 14% in *BRAF*-negative cases; *P*=0.03). By

contrast, the two mutations did not result associated in either the control group or the overall population in analysis, probably due to the small number of *TERT* mutated cases in the control group.

In the overall cohort of 121 samples, the coexistence of *TERT* promoter mutations and *BRAF* V600E mutation was significantly associated with older age, TCv histotype, advanced stage, and worse outcome. *TERT* mutation alone remained independently associated with the presence of distant metastases. Within the DM-PTCs group, *TERT* mutations were associated with older age, either alone or in association with *BRAF* V600E. By contrast, in the control-PTCs group, the *BRAF* V600E was associated with older age, either alone or in association with *TERT* promoter mutations (Table 5).

Effect of *TERT* promoter mutations on thyroid cancer-specific survival

Next, we investigated the effect of *TERT* promoter mutations on the survival of PTCs patients. The Kaplan–Meier survival analysis showed that the presence of *TERT* promoter mutations was strongly associated with increased disease-specific mortality in the overall PTCs cases analyzed (log-rank *P*=0.001; Fig. 2A). However, the same analysis performed within the DM-PTCs showed no statistically significant difference in the survival curves (Fig. 2B).

When we analyzed the survival curves by *TERT* mutation, *BRAF* mutation, and their coexistence status in the overall PTCs cases, we found that PTCs with either

Figure 2

(A and B) Kaplan–Meier curves of thyroid carcinoma-specific survival by *TERT* mutation status in the overall PTCs cases analyzed in this study ($n=121$, A) and in DM-PTCs only ($n=43$, B). (C and D) Kaplan–Meier curves of thyroid carcinoma-specific survival by *TERT* mutation, *BRAF* mutation, and their coexistence status in the overall PTCs cases analyzed in this study ($n=121$, C) and in DM-PTCs only ($n=43$, D). Survival curves were compared by log-rank test, using the WT as reference. The $P<0.05$ were considered as threshold for significance. The P values of the comparison between *TERT*+*BRAF*-double mutants and *TERT* single-mutant was nonsignificant (0.90), while the comparison between *TERT*+*BRAF*-double mutants and *BRAF* single-mutant was significant (0.02).

only *TERT* or both *TERT* and *BRAF* mutations showed a significantly lower survival probability compared with WT PTCs (log-rank $P=0.02$ and $P=0.03$ respectively), while *BRAF* mutation status alone did not affect the survival (Fig. 2C). Again, we did not observe any significant difference in the survival curves when performing the same analysis within the DM-PTC group (Fig. 2D).

Finally, we evaluated the effect on survival of the co-occurrence of both *BRAF* and *TERT* promoter mutations as compared with the occurrence of *BRAF* or *TERT* promoter single mutants (Fig. 2C).

We did not observe a significant effect of the *BRAF* V600E mutation on the survival curve of *TERT* mutated tumors ($P=0.90$). By contrast, the presence of *TERT* mutations strongly decreased the survival probability of *BRAF* V600E mutated PTC patients ($P=0.02$).

Discussion

Well-differentiated PTCs are usually indolent lesions with low rate of growth and low metastatic potential. Nonetheless, a small but significant percentage of PTCs (5–10%) behave aggressively, developing metastases and possibly leading to patient's death (2, 3, 29). The identification of prognostic markers able to discriminate aggressive PTCs from those with an indolent course would be of great relevance to better tailor the therapeutic approach and the long-term surveillance, avoiding overtreatment (34). The relative rarity of aggressive PTCs and the slow course of this disease make it difficult to collect large cohorts of tumors with distant metastases, which is necessary to obtain a more exhaustive comprehension of the genetic and biological processes at the basis of the metastatic

spreading of these tumors and to improve the current prognostic capacity in the diagnosis of PTCs.

In this study, we analyzed a cohort of 43 DM-PTCs and we showed that mutation in the *TERT* promoter is a frequent event in metastatic tumors. Indeed, the occurrence of *TERT* mutations was more than 3.5-fold higher (33% vs 9%) in the group of DM-PTCs as compared with the control group of nonaggressive tumors. The high prevalence of these alterations in aggressive PTCs indicates that *TERT* promoter mutations, in particular the C228T mutation, could be a promising marker for risk stratification of PTC patients.

This is one of the first studies in which the occurrence of *TERT* promoter mutations is directly correlated with the presence of distant metastases in PTC. Two recent studies (27, 33) have analyzed *TERT* promoter mutations in large PTC sets, which included also a subset of patients who developed metastases at distant sites (36 and 22 cases respectively). Melo *et al.* (27) described not only a strong association between the presence of *TERT* promoter mutations and a low grade of differentiation of the tumors, but also a significant association between the presence of the mutations and the development of distant metastases in well-differentiated thyroid cancer, including PTCs and follicular thyroid carcinoma (FTCs) cases. This association was not significant in the PTCs subgroup only, albeit it tended to significance. In another study, Xing *et al.* (33) found a significantly higher frequency (45%) of the C228T mutation in 22 PTCs with distant metastases as compared with the one detected in PTCs without distant metastases (10%). Our observation in a larger DM-PTC set extends and supports these data, confirming that *TERT* promoter mutations are major genetic features of PTCs with metastatic behavior, and sustaining the hypothesis that these alterations may play a relevant role in determining the progression of thyroid cancer.

In our analysis, *TERT* promoter mutations were also associated with an older age at diagnosis and with the TCV-PTC histotype, which is a more aggressive variant compared with classic PTCs and follicular-variant PTCs, in agreement with previously published data (24). Furthermore, we observed a strong association between the presence of these mutations and increased disease-related mortality when considered the overall cohort of samples in analysis.

Taken together, these data indicate that *TERT* promoter mutations, in particular the C228T mutation, are major genetic events during the acquisition of metastatic potential in thyroid cancer. However, the

fact that the disease-related mortality within the DM-PTCs was not related with *TERT* mutations seems to suggest that once the metastatic behavior has been acquired, the *TERT* status does not affect the outcome of the patients.

It has been reported that *TERT* promoter mutations are able to increase the transcriptional activity of the promoter (12, 13, 18), and that *TERT*-mutated tumors express higher level of *TERT* mRNA compared with WT-tumors (17, 18, 19, 20, 35). *TERT* is a reverse transcriptase involved in the maintenance of the integrity of telomere length. In normal, actively proliferating cells, the progressive shortening of telomeres leads eventually to replicative senescence. In this regard, it is conceivable that the acquisition of features leading to *TERT* activation is an important event during cancer progression, as it allows tumor cells to avoid proliferation limitation and to acquire immortalization (21). Furthermore, telomere-independent activities of *TERT* have been reported and several studies showed that this protein is engaged in processes such as regulation of DNA damage response, repression of apoptosis, regulation of chromatin state, and enhanced cell proliferation that are important for cancer progression (36, 37, 38, 39, 40).

In the aggressive DM-PTCs group, we found that C228T mutation tended to be prevalent in tumors harboring also the BRAF V600E mutation, while this association was not evaluable in control-PTCs due to the small number of *TERT*-mutated cases. Association between *TERT* and BRAF mutation has been previously reported (24, 26, 27, 33) and it has been proposed that their coexistence may define a more aggressive subgroup of PTC (33). Our results suggest that *TERT* promoter mutations are associated with the presence of distant metastases independently from the BRAF status, which is not predictive, alone or in association with *TERT* mutations, with aggressive behavior in our cohort, as previously described (31, 32).

Moreover, we found that the occurrence of *TERT* promoter mutations decrease the disease-specific survival of BRAF V600E mutated tumors, while the presence of BRAF V600E mutation did not affect significantly the outcome of *TERT*-mutated PTCs.

In conclusion, *TERT* promoter mutations, and in particular *TERT* C228T, were found to be strongly associated with tumor progression and development of distant metastases in PTCs. When confirmed by larger, prospective studies on consecutive cases, these findings may help in elucidating the usefulness of *TERT* as a prognostic marker of aggressive behavior in this cancer.

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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