Tall cell papillary thyroid carcinoma: new diagnostic criteria and mutations in *BRAF* and *TERT*

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Abstract

The tall cell (TC) variant of papillary thyroid carcinoma (PTC) has an unfavorable prognosis. The diagnostic criteria remain inconsistent, and the role of a minor TC component is unclear. Molecular diagnostic markers are not available; however, there are two potential candidates: *BRAF V600E* and telomerase reverse transcriptase (*TERT*) promoter mutations. Using a novel approach, we enriched a collective with PTCs that harbored an adverse outcome, which overcame the limited statistical power of most studies. This enabled us to review 125 PTC patients, 57 of which had an adverse outcome. The proportion of TCs that constituted a poor prognosis was assessed. All of the tumors underwent sequencing for *TERT* promoter and *BRAF V600E* mutational status and were stained with an antibody to detect the *BRAF V600E* mutation. A 10% cutoff for TCs was significantly associated with advanced tumor stage and lymph node metastasis. Multivariate analysis showed that TCs above 10% were the only significant factor for overall, tumor-specific, and relapse-free survival. Seven percent of the cases had a *TERT* promoter mutation, whereas 61% demonstrated a *BRAF* mutation. The presence of TC was significantly associated with *TERT* promoter and *BRAF* mutations. *TERT* predicted highly significant tumor relapse ($P < 0.001$). PTCs comprised of at least 10% TCs are associated with an adverse clinical outcome and should be reported accordingly. *BRAF* did not influence patient outcome. Nevertheless, a positive status should encourage the search for TCs. *TERT* promoter mutations are a strong predictor of tumor relapse, but their role as a surrogate marker for TCs is limited.

Key Words

- ▶ papillary thyroid carcinoma
- ▶ tall cell
- ▶ diagnostic criteria
- ▶ prognosis
- ▶ *BRAF*
- ▶ *TERT*

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Introduction

Thyroid carcinoma is classified into five different morphological groups: papillary, follicular, medullary, poorly differentiated, and anaplastic/undifferentiated (DeLellis et al. 2004). These endocrine cancers account for ~1% of all malignancies (Schlumberger 1998). Papillary thyroid

carcinomas (PTCs) are among the most curable cancers, and total thyroidectomy followed by adjuvant radioiodine administration cures almost all PTC patients. Patient prognosis depends on age, sex, tumor size, and extra-thyroidal extension, the latter two of which are reflected

in the UICC TNM staging system (Sobin *et al.* 2009). On a morphologic basis, PTCs are subdivided into different subtypes, as recognized by the WHO classification (DeLellis *et al.* 2004). Nevertheless, this subclassification has little clinical significance, because the different subtypes do not add independent prognostic value. As a result, the vast majority of PTCs are not routinely subclassified. A grading system for PTCs has been proposed that includes nuclear atypia, tumor necrosis, and vascular invasion as signs of a poorly differentiated PTC (Akslen & LiVolsi 2000). However, most of these tumors would nowadays probably be classified as poorly differentiated thyroid carcinomas (Volante *et al.* 2007). Different staging systems for PTCs have been introduced. Unfortunately, they are very complex, and as a consequence, they are not used on a daily basis in most institutions. In fact, it is very difficult to predict which patients will suffer from recurrent disease or death from PTC and which will be cured by the combination of surgery and radioiodine treatment (Nikiforov *et al.* 2012).

Among the different PTC subtypes, the tall cell variant (TCV) is known to have a more aggressive clinical behavior (LiVolsi 2010). However, the morphological criteria for the diagnosis of this entity is not clearly defined by the WHO ('The TCV is composed predominantly of cells whose heights are at least three times their widths.'), and the percentage of tall cells (TCs) needed in a given tumor is defined variably in the literature (Ostrowski & Merino 1996, DeLellis *et al.* 2004).

The oncogenic *BRAF* V600E mutation occurs in about 40–45% of PTCs (Nikiforov *et al.* 2012). It activates the MAPK signaling pathway in human cancer and has been shown to correlate with aggressive features in PTC, including extrathyroidal extension, lymph node metastasis, and the TCV (Kim *et al.* 2012, Koperek *et al.* 2012, Li *et al.* 2012, Xing *et al.* 2013). An increasing number of studies that include meta-analyses have been able to demonstrate an association between *BRAF* status and aggressive tumor behavior (Elisei *et al.* 2012, Kim *et al.* 2012, Li *et al.* 2012, Xing *et al.* 2013). Other studies, however, could not confirm this data, which has resulted in uncertainty about the value of *BRAF* in PTC (Ito *et al.* 2009, Cheng *et al.* 2011, Eloy *et al.* 2011, Koperek *et al.* 2012, Sancisi *et al.* 2012, Barbaro *et al.* 2014). One reason for this could be the fact that an adverse patient outcome with multiple tumor relapses and eventually death resulting from PTC is a rare event. As a matter of fact, only about 5% of PTCs will have an adverse outcome in consecutive clinicopathological series, and this has led to very small groups of high-risk tumors with limited

statistical power in different studies (Handkiewicz-Junak *et al.* 2010).

Reactivation of the telomerase reverse transcriptase (*TERT*) gene, which encodes for the catalytic subunit of telomerase, is implicated in tumorigenesis and cell immortalization. The two promoter mutations C228T and C250T were recently reported in human melanomas and other human cancer types, including thyroid cancer (Huang *et al.* 2013, Liu *et al.* 2013a, Vinagre *et al.* 2013). They are novel mechanisms in tumor biology that generate *de novo* consensus-binding motifs for E-twenty-six transcription factors, increase the activity of the *TERT* promoter, and have potential clinical applications and prognostic value (Huang *et al.* 2013, Landa *et al.* 2013, Liu *et al.* 2013b).

The purpose of this study was to analyze the prevalence, histomorphology, and follow-up data of a cohort of PTC patients enriched for patients with a known adverse clinical outcome (ACO) based on a population of 1.3 million inhabitants over a 16-year period and to determine the threshold of TC within a given PTC that could impact patient outcome. Furthermore, we sequenced *BRAF* and *TERT* promoter mutations in all tumors and evaluated whether they can serve as surrogate markers for TCV and an ACO.

Material and methods

Because radioiodine treatment is the standard therapy for patients suffering from recurrent thyroid cancer, virtually all such patients are seen in the department of nuclear medicine. Three such departments (at the University Hospital Zürich, the City Hospital Triemli, and the Cantonal Hospital Winterthur) serve the 1.3 million inhabitants in the canton of Zürich (Statistics Do 2011). Fifty-seven patients were identified in these departments who had thyroid surgery between 1990 and 2006 and had an ACO. An ACO was defined as more than one tumor recurrence or tumor-related death. Sixty-eight age-, stage- and sex-matched patients (without recurrence) treated for PTC at the University Hospital Zürich were used as the control group (CG; Table 1). Tumors with a size of <1 cm were excluded from this study, because these microcarcinomas show a very indolent clinical course (Baloch & LiVolsi 2006) and the significance of TC in this setting is beyond the scope of the study. This approach significantly enhanced the number of patients with an ACO. Twenty-one of the patients had lymph node metastases, whereas 104 patients were node negative.

Archival pathology specimens of the thyroid tumors were reevaluated according to the WHO classification by

Table 1 Clinical and pathological characteristics between the adverse clinical outcome (ACO) group and the control group (CG); χ^2 test

	Total (n)	ACO	CG	P value
Age (years)				
<48	69 (55.2%)	32 (25.6%)	37 (29.6%)	0.859
≥48	56 (44.8%)	25 (20%)	31 (24.8%)	
Sex				
Male	34 (27.2%)	20 (16%)	14 (11.2%)	0.106
Female	91 (72.8%)	37 (29.6%)	54 (43.8%)	
pT				
pT 1–2	55 (44%)	18 (14.4%)	37 (29.6%)	0.012
pT 3–4	70 (56%)	39 (31.2%)	31 (24.8%)	
LN				
No metastasis	104 (83.2%)	38 (30.4%)	66 (52.8%)	0.001
Metastasis	21 (16.8%)	19 (15.2%)	2 (1.6%)	
TC (%)				
TC <10	77 (61.6%)	21 (16.8%)	56 (44.8%)	0.00001
TC ≥10	48 (38.4%)	36 (28.8%)	12 (9.6%)	
BRAF total ^a				
WT	49 (39.2%)	17 (13.6%)	32 (25.6%)	0.066
Mutated	76 (60.8%)	40 (32.0%)	36 (28.8%)	
TERT				
WT	102 (92.7%)	45 (40.9%)	57 (51.8%)	0.002
Mutated	8 (7.3%)	8 (7.3%)	0 (0%)	

^aResult of *BRAF* testing by pyrosequencing or by the VE1 antibody when no *BRAF* mutational testing by pyrosequencing was possible.

three pathologists who were blinded to the clinicopathological data (M D, A S, and A P), and a consensus diagnosis was rendered (DeLellis et al. 2004). The tumor stage was reclassified based on the seventh TNM classification (Sobin et al. 2009). TCs were defined as cells that were more than three times as high as they were wide, often with an eosinophilic cytoplasm (Nikiforov et al. 2012). A few examples and pitfalls are shown in Fig. 1. Although the so-called ‘tram-track’ pattern, especially at low power, is a very helpful feature, not all areas showing this growth pattern are TCs. Furthermore, areas that exhibit a classic PTC growth pattern can be comprised of true TCs, which thus makes it mandatory for the pathologist to examine all tumors at high magnification. Nuclear stratification, intranuclear inclusions, and characteristic coffee bean infoldings of the nuclear membrane were frequently seen (Nikiforov et al. 2012).

The percentage of TCs was recorded semiquantitatively in each tumor in 10% increment steps, ranging from 0 to 100%; the lowest recorded amount of TC in a given tumor was therefore 10%. All available slides were assessed in each tumor (6.1 ± 4.5 slides per case in the CG and 5.0 ± 4.5 slides in the ACO group). Tumors that had anaplastic or poorly differentiated areas, as defined by the Turin criteria, were

excluded from the study (Volante et al. 2007) and have been reported elsewhere (Dettmer et al. 2011, 2012).

DNA was extracted using the DNeasy Blood and Tissue kit, and all of the samples underwent *BRAF* pyrosequencing mutation analysis on the PyroMark Q24 System (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. The primers had the following sequence: forward: 5′-TGAAGACCTCACAGTAAAAATA-GG-3′; reverse: 5′-TCCAGACAACCTGTTCAAAGTAT-3′; sequencing primer: 5′-TGATTTTGGTCTAG-CTACA-3′. An allelic fraction of ≥10% mutant alleles was defined as positive for the mutation.

A tissue microarray (TMA) was constructed and stained with the *BRAF* V600E mutation-specific antibody as previously described (Koperek et al. 2012, Boos et al. 2013). A tumor was considered positive when a distinct, homogenous immunoreaction was seen in the cytoplasm of all of the cancer cells. Immunohistochemistry (IHC) and molecular analysis were performed on the same tissue block.

TERT promoter mutations were assessed by PCR, followed by Sanger Sequencing as described previously (Liu et al. 2013a).

The sample distribution was analyzed using the Kolmogorov–Smirnov test. χ^2 , Kendall’s tau_b, Kruskal–Wallis, Kaplan–Meier and COX regression tests were performed using SPSS version 21.0 (SPSS, Inc., IBM, Chicago, IL, USA). A *P* value of <0.05 was considered statistically significant. The study was approved by the Cantonal Research Ethics Board, Zürich (STV 28-2006).

Results

Fifty-seven PTC patients with ACO and 68 PTC patients in the CG were analyzed. The characteristics of the study population are summarized in Tables 1 and 2. The means $\pm$ s.d. for the different follow-up times in months were: overall survival (OS): 81.97 ± 48.42 ; tumor-specific survival (TSS): 81.97 ± 48.42 ; relapse-free survival (RFS): 51.78 ± 50.97 ; CG: 81.88 ± 48.5 ; and ACO: 81.5 ± 49.5 . There were 68 classic PTCs, 47 follicular variants of PTC (FVPTC), and ten PTCs of special subtypes (three diffuse sclerosing, two columnar cell, two solid, two warthin-like, and one oncocytic variant). Seventy-seven PTCs did not display any detectable TCs, 27 PTCs showed >10% but <50% TCs, and 21 tumors had ≥50% TCs. The distribution of TCs and the important clinical endpoints OS, TSS, and RFS are summarized in Table 2 and Supplementary Table 1, see section on supplementary data given at the end of this article.

Figure 1

(A) Papillary cancer, tall cell variant: tumor cells are at least three times as high as they are wide and show an eosinophilic cytoplasm and elongated follicles (HE, 200 $\times$). (B) Same tumor as (A). Papillary cancer, tall cell variant: cells arranged in a 'tram-track' pattern with eosinophilic cytoplasm (HE, 400 $\times$). (C) Papillary cancer, tall cell variant: papillary growth pattern with delicate branching of papillae (HE, 200 $\times$). (D) Same tumor as (C). Papillary cancer, tall cell variant: cells growing in a classic papillary fashion

(HE, 400 $\times$). (E) Not all cells in these tumors are at least three times high as they are wide. However, tumor cells growing in the 'tram-track' pattern should trigger a careful search for tall cells (HE, 200 $\times$). (F) Same tumor as (E). Tumor cells are cut tangential to their base (upper). Cells can only be assessed where the basal membrane is seen (lower). These tumor cells are classic tall cells, often with nuclear grooves and elongated follicles (HE, 400 $\times$).

The tumors comprising of 10–50% TCs showed a presence of TCs predominantly at the invasive front, whereas tumors harboring $\geq 50\%$ TC showed a presence of TCs in the center of the tumor as well.

Tall cells

The presence of TCs showed significant correlation with patient survival, relapse, and tumor stage.

Clinicopathological data for the tumors were also correlated with the different thresholds for TC presence in the tumors, and ROC curves were calculated to assess the best cutoff (Supplementary Fig. 1 and Table 2, see section on supplementary data given at the end of this article). TCs of $>10\%$ correlated with lymph node metastasis ($P < 0.001$) but not with organ metastases ($P < 0.223$). TCs of $\geq 50\%$ did not correlate with lymph node metastasis ($P < 0.141$) nor with organ metastasis ($P < 0.601$).

Table 2 Clinicopathological characteristics of the study population for papillary thyroid carcinoma (PTC) with different cutoffs for the tall cell variant (TCV) of PTC; χ^2 test

	Total (n)	TC <10% (n)	TC ≥10% (n)	P value	TC <50% (n)	TC ≥50% (n)	P value
Age (years)							
<48	69 (55.2%)	44 (35.2%)	25 (20%)	0.586	62 (49.6%)	7 (5.6%)	0.032
≥48	56 (44.8%)	33 (26.4%)	23 (18.4%)		42 (33.6%)	14 (11.2%)	
Sex							
Male	34 (27.2%)	17 (13.6%)	17 (13.6%)	0.147	26 (20.8%)	8 (6.4%)	0.282
Female	91 (72.8%)	60 (48%)	31 (24.8%)		78 (62.4%)	13 (10.4%)	
pT							
pT 1–2	55 (44%)	40 (32%)	15 (12%)	0.027	51 (40.8%)	4 (3.2%)	0.015
pT 3–4	70 (56%)	37 (29.6%)	33 (26.4%)		53 (42.4%)	17 (13.6%)	
LN							
No metastasis	104 (83.2%)	74 (59.2%)	30 (24%)	0.0001	90 (72%)	14 (11.2%)	0.049
Metastasis	21 (16.8%)	3 (2.4%)	18 (14.4%)		14 (11.2%)	7 (5.6%)	
OS							
Censored	111 (88.8%)	75 (60%)	36 (28.8%)	0.0001	97 (77.6%)	14 (11.2%)	0.002
OS							
Death	14 (11.2%)	2 (1.6%)	12 (9.6%)		7 (5.6%)	7 (5.6%)	
TSS							
Censored	118 (94.4%)	77 (61.6%)	41 (32.8%)	0.001	102 (81.6%)	16 (12.8%)	0.001
TSS							
Death	7 (5.6%)	0 (0%)	7 (5.6%)		2 (1.6%)	5 (4%)	
RFS							
Censored	68 (54.4%)	56 (44.8%)	12 (9.6%)	0.0001	66 (52.8%)	2 (1.6%)	0.0001
RFS							
Relapse	57 (45.6%)	21 (16.8%)	36 (28.8%)		38 (30.4%)	19 (15.2%)	

TC, tall cells; OS, overall survival; TSS, tumor specific survival; RFS, relapse free survival.

Kaplan–Meier survival analysis showed significant reduction in OS, TSS, and RFS for tumors that had more than 10% TCs (each: log-rank $P<0.001$). To further determine what proportion of $\geq 10\%$ TCs impacts patient prognosis, we subcategorized this group into two groups, namely, 10–50 and $\geq 50\%$ TC area. There were no significant differences between the two groups with

regards to OS, TSS, or RFS, but in the group with 10–50% TCs, there was a tendency for less aggressive behavior as compared to the group with $\geq 50\%$ TC area (Fig. 2).

Multivariate Cox regression analysis was performed using patient age, sex, tumor stage, and TC tumor area ($<10\%$ vs $\geq 10\%$) for OS, RFS, and TSS. For RFS, TC $\geq 10\%$ was the only parameter that was significant ($P<0.0001$,

Figure 2

(A) Kaplan–Meier, overall survival (OS); tall cells $<10\%$ and $\geq 10\%$; log-rank $P<0.001$. TC $<10\%$: mean $\pm$ S.E.M. 200.07 ± 7.37 ; 95% CI 185.62, 214.52; TC $\geq 10\%$: mean $\pm$ S.E.M. 152.14 ± 14.90 ; 95% CI 122.93, 181.34. (B) Kaplan–Meier, relapse-free survival; tall cells $<10\%$ and $\geq 10\%$; log-rank $P<0.001$. TC $<10\%$: mean $\pm$ S.E.M. 150.98 ± 10.9 ; 95% CI 129.63, 172.32. TC $\geq 10\%$: mean $\pm$ S.E.M. 40.20 ± 7.0 ; 95% CI 26.49, 53.91. (C) Kaplan–Meier, OS; tall cells $<10\%$ vs $\geq 10\%$ vs $\geq 10\%$ vs $\geq 50\%$; log-rank $P<0.518$. TC $<10\%$: mean $\pm$ S.E.M. 200.07 ± 7.37 ; 95% CI

185.62, 214.52. TC $\geq 10\%$ vs $<50\%$: mean $\pm$ S.E.M. 162.48 ± 14.94 ; 95% CI 133.20, 191.76. TC $\geq 50\%$: mean $\pm$ S.E.M. 138.85 ± 20.18 ; 95% CI 99.30, 178.40.

(D) Kaplan–Meier, relapse-free survival; tall cells $<10\%$ vs $\geq 10\%$ vs $<50\%$, log-rank $P<0.001$; and tall cells $\geq 10\%$ vs $\geq 50\%$; log-rank $P<0.379$. TC $<10\%$: mean $\pm$ S.E.M. 150.98 ± 10.9 ; 95% CI 129.63, 172.32.

TC $\geq 10\%$ vs $<50\%$: mean $\pm$ S.E.M. 50.32 ± 10.87 ; 95% CI 29.01, 71.63.

TC $\geq 50\%$: mean $\pm$ S.E.M. 32.43 ± 8.58 ; 95% CI 15.62, 49.24.

Exp(B)=3.6). For OS, the factors that were significant were age ($P<0.010$, Exp(B)=6.3), and TC $\geq 10\%$ ($P<0.001$, Exp(B)=12.9). The analysis could not be performed for TSS, because no patient in the TC $<10\%$ group died. However, when the analysis for TSS was performed using the threshold of TC $\geq 50\%$, it was significant ($P<0.008$, Exp(B)=9.3). The Kruskal–Wallis test revealed a significant association between the number of relapses and the percentage of TCs found in a given tumor ($P<0.001$; [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article).

BRAF

Pyrosequencing analysis detected *BRAF V600E* in 72 PTC cases (61%), whereas 46 tumors (39%) were negative. All of the mutations were *V600E*; other *BRAF* mutations were not detected. The sensitivity was higher as compared to

the mutation-specific antibody, which detected the mutation in 63 cases (50.4%) and which was negative in the other 62 patients (49.6%). The allelic fraction of *BRAF* (29.4 ± 13.5 , range 10–46) mutation did not correlate with patient outcome. Pyrosequencing was more sensitive in nine discrepant cases, and it detected the *BRAF* mutation when the antibody was negative. Two of these cases had subsequent lymph node metastases, which were then positive for *BRAF* by the antibody. Only two tumors that showed a positive IHC for *V600E* were negative by pyrosequencing. In summary, there was an excellent, highly significant correlation between both detection methods ($P<0.001$, $r=0.600$; [Supplementary Table 2](#)).

BRAF status was not associated with patient outcome in the Kaplan–Meier analysis or the Cox regression multivariate analysis ([Fig. 3](#)). There were no associations of *BRAF* mutations with lymph node or organ metastases.

Figure 3

The influence of *BRAF* (A, B and C) and *TERT* promoter mutations (D, E and F) on overall survival, tumor-specific survival (TSS), or relapse-free survival in patients with PTC; all log-rank test. (A) Kaplan–Meier, overall survival; *BRAF* wt: mean $\pm$ s.e.m. 191.75 ± 11.04 ; 95% CI 170.11, 213.38; *BRAF V600E*: mean $\pm$ s.e.m. 175.60 ± 10.30 ; 95% CI 155.40, 195.79. (B) Kaplan–Meier, TSS; *BRAF* wt: mean $\pm$ s.e.m. 210.22 ± 4.65 ; 95% CI 201.10, 219.33; *BRAF V600E*: mean $\pm$ s.e.m. 187.34 ± 9.30 ; 95% CI 169.11, 205.58. (C) Kaplan–Meier, relapse-free survival; *BRAF* wt: mean $\pm$ s.e.m. 121.822 ± 12.76 ; 95% CI 96.82, 146.82; *BRAF V600E*: mean $\pm$ s.e.m. 96.99 ± 12.08 ; 95% CI 73.31, 120.67.

(D) Kaplan–Meier, overall survival; *TERT* promoter wt: mean $\pm$ s.e.m. 196.64 ± 8.84 ; 95% CI 169.31, 203.96; *TERT* promoter mutated: mean $\pm$ s.e.m. 107.18 ± 19.80 ; 95% CI 68.37, 145.99. (E) Kaplan–Meier, TSS; *TERT* promoter wt: mean $\pm$ s.e.m. 200.60 ± 6.39 ; 95% CI 188.08, 213.11; *TERT* promoter mutated: mean $\pm$ s.e.m. 127.63 ± 13.38 ; 95% CI 101.40, 153.86. (F) Kaplan–Meier, relapse-free survival; *TERT* promoter wt: mean $\pm$ s.e.m. 101.34 ± 9.09 ; 95% CI 83.52, 119.16; *TERT* promoter mutated: mean $\pm$ s.e.m. 16.13 ± 6.15 ; 95% CI 4.07, 28.18.

Table 3 Tall cells, *BRAF*, and *TERT* promoter mutation status of the study population for papillary thyroid carcinoma (PTC) with different cutoffs for the TCV; χ^2 test. *BRAF* pyro, *BRAF* V600E mutational status as detected by pyrosequencing; *BRAF* antibody, *BRAF* V600E mutational status as detected by the mutation-specific VE1 antibody for *BRAF* V600E; *BRAF* total, *BRAF* V600E mutational status as detected by pyrosequencing when available and otherwise by the mutation-specific VE1 antibody for *BRAF* V600E

	Total (n)	TC < 10% (n)	TC ≥ 10% (n)	P value	TC < 50% (n)	TC ≥ 50% (n)	P value
<i>BRAF</i> pyro							
WT	46 (39%)	37 (31.4%)	9 (7.6%)	0.001	41 (34.7%)	5 (4.2%)	0.143
Mutated	72 (61%)	35 (29.7%)	37 (31.4%)		56 (47.5%)	16 (13.6%)	
<i>BRAF</i> antibody							
WT	62 (49.6%)	45 (36%)	17 (13.6%)	0.017	53 (42.4%)	9 (7.2%)	0.633
Mutated	63 (50.4%)	32 (25.6%)	31 (24.8%)		51 (40.8%)	12 (9.6%)	
<i>BRAF</i> total							
WT	49 (39.2%)	39 (31.2%)	10 (8%)	0.001	44 (35.2%)	5 (4%)	0.088
Mutated	76 (60.8%)	38 (30.4%)	38 (30.4%)		60 (48%)	16 (12.8%)	
<i>TERT</i>							
WT	102 (92.7%)	65 (59.1%)	37 (33.6%)	0.004	89 (80.9%)	13 (11.8%)	0.0003
Mutated	8 (7.3%)	1 (0.9%)	7 (6.4%)		2 (1.8%)	6 (5.5%)	

TCs and *BRAF*

Sixteen out of 21 patients (76%) had the *BRAF* mutation when the 50% cutoff for the TCV was applied. This went up to 79% (38 out of 48 patients) when the 10% cutoff for the TCV diagnosis was applied. The amount of TC within a given tumor was significantly associated with *BRAF* status ($P < 0.002$; Table 3, Supplementary Table 1).

The allelic fraction as determined by pyrosequencing was influenced by the amount of TC within a tumor ($P < 0.034$, $r = 0.156$; Kendall's tau_b). There was no significant association between *BRAF* status and an ACO on univariate or multivariate analysis for either the 10% or for the 50% cutoff when we analyzed only the TC tumors (Table 1, Supplementary Tables 2 and 3).

TCs, *BRAF*, and *TERT*

TERT promoter mutations were detected in eight tumors (7.3%). The C250T mutation was found in three tumors, and the C228T mutation was found in five. Six patients with *TERT* promoter mutations were also *BRAF* mutated, whereas two were not ($P < 0.49$). TC morphology correlated significantly with this mutation ($P < 0.0001$, $r = 0.308$); six of these tumors had TC morphology in $\geq 50\%$, whereas one had 30% TC and one had none. All eight patients with the *TERT* promoter mutation had a tumor relapse ($P < 0.001$). Tumors that were *TERT* promoter mutated and *BRAF* mutated behaved significantly worse than *BRAF* mutated tumors alone did (log-rank $P < 0.019$; Table 1 and Supplementary Table 2).

TERT promoter mutations, together with pT stage, remained an independent predictor of tumor relapse in

a multivariate analysis (*TERT* promoter mutation: $P < 0.005$, Exp(b) = 3.18; pT stage: $P < 0.019$, Exp(b) = 1.58). However, when TCs were included in the analysis, they remained the single independent predictor of RFS ($P < 0.0001$, Exp(b) = 3.04), whereas the other two factors dropped out (Supplementary Table 3).

The numbers of some parameters do not add up to the total number of patients included in the study because of dropouts in DNA quality and the consecutive failure of mutational testing or because of dropout of cases on the TMA.

Discussion

In this study, we analyzed a large cohort of PTC and TCV with an ACO, delineated the proportion of TC that is needed to determine patient outcome, and assessed the role of *BRAF* and *TERT* promoter mutations in this setting. In the study, a 10% cutoff of TC defines a detrimental prognosis. *BRAF* mutation status does not correlate with adverse outcome, but it is more frequent in TCV than it is in classical PTC. *TERT* mutations are rare, but they are associated with a very aggressive course.

Previous clinicopathological studies on TCV PTC analyzed small groups of tumors with ACO, which thereby limited their statistical power (Ostrowski & Merino 1996, LiVolsi 2010). In this study, the number of patients with ACO was increased to a relatively large sample size via the identification of patients in nuclear medicine departments. This approach increased the statistical power in determining a cutoff of TC leading to poor prognosis. This patient selection is, on the other hand, also a limitation of the study, because it does not reflect the normal patient

population. We can therefore not exclude the possibilities that we overestimated the effect of TC in tumors with an ACO and that TCs are much more frequently encountered in a consecutive series of patients when they do not account for an ACO. However, in our age-, stage- and sex-matched cohort, this was not the case.

The TCV PTC is a distinct entity of thyroid tumors that is associated with an unfavorable patient outcome. The threshold of proportion of TCs in a given carcinoma to allow for the diagnosis of TCV is poorly defined and differs in the literature from no information at all to 10–75% (Sobrinho-Simoes *et al.* 1989, Ostrowski & Merino 1996, Akslen & LiVolsi 2000, DeLellis *et al.* 2004, LiVolsi 2010). Our results confirm that PTCs comprising more than 50% TCs have an ACO. Although this result was expected, it settles the finding in a robust study population. Survival analysis showed that there was a clear difference between TC and non-TC PTCs using this 50% cutoff. However, the results were more interesting when a cutoff at 10% was introduced, because the influence of a minor TC component is unclear and controversial (LiVolsi 2010, Nikiforov *et al.* 2012). To exclude the possibility that the group of PTCs comprising of $\geq 50\%$ TCs skewed the analysis and that a TC component of 10–50% did not severely affect patient outcome, we separated the tumors into three groups, $<10\%$ TCs, 10–50% TC, and $\geq 50\%$ TC. Kaplan–Meier analysis revealed that the presence of at least 10% TCs in a PTC significantly impacted the patient outcome for OS, TSS, and RFS. Moreover, a PTC with $\geq 10\%$ TCs is the only significant factor in a multivariate analysis, which included age, sex, and tumor stage, for ACO.

It has already been proposed by Sobrinho and others (Sobrinho-Simoes *et al.* 1989, LiVolsi 2010) that it is important for pathologists to report the number of TCs seen in a given tumor, few as they may be. Two studies have suggested that a minor TC component may determine patient outcome, but they were not able to confirm this in a univariate or multivariate analysis respectively (Beninato *et al.* 2013, Ganly *et al.* 2013). The first study (Beninato *et al.* 2013) conducted a review on cases that had been diagnosed as TCV or PTC with a minor TC component. With such an approach, we would have missed most of the TCVs in this study. In fact, only three PTCs consisting exclusively of TCs were diagnosed as TCVs in the original pathology report. All of the remaining PTCs were originally diagnosed as either classical PTCs or FVPTCs, even when a threshold of 50% was applied. These underdiagnoses could be a result of the rarity of this condition and a lack of awareness on the part of the

surgical pathologists, on the one hand, or a result of the ambiguous definition of the condition, on the other hand. The second study (Ganly *et al.* 2013) defined TCs as cells with a height that is twice the size of their width and therefore included as TCVs tumors that would not be included as TCVs in this work. Our results indicate that the strict application of the 2004 WHO definition of TCs as cells with a length that is thrice their width better stratifies patients (DeLellis *et al.* 2004). That study also had only a few patients with adverse events. Because of these reasons, it was probably therefore not significant in the multivariate analysis.

In this study, it is evident that the presence of a TC component of 10–50% significantly and independently affects patient prognosis and that there is no statistical difference between the group with a 10–50% TC component and that with a $\geq 50\%$ TC component. The PTCs with 10–50% TCs would be regarded as conventional PTCs or as PTCs with TC features by most authors (Ghossein & Livolsi 2008, LiVolsi 2010). At this point, it is important to specify that we examined approximately six tumor blocks per patient and that examining only one block per tumor would have lowered the rate of detection of TCV by 11% when using the 10% cutoff. To our knowledge, no image analysis tool is available that would be able to identify TCs within a PTC and to quantify their amount in a given tumor.

The Kaplan–Meier survival analysis in this study suggests that there might be a difference between the tumors that harbor a 10–50% TC component vs a $\geq 50\%$ TC component, with the former showing a tendency for less aggressive behavior. We propose two potential explanations for this finding. First, there could be a limitation in the morphological detection of TC areas in the sub 10% range that could lead to these tumors being categorized in the 10–50% TC group, which would thereby account for the intermediate results in the Kaplan–Meier analysis. This also implies that the greater the TC component that was identified, the higher the possibility that the tumor was truly a TCV of PTC. The second explanation for this finding is that there could be a gradual increase of TC that leads to a gradually increased aggressiveness. Further support for the second explanation can be found the comparison of TCV to other solid carcinomas. Different subclones in tumors play an important role in the formation of tumor progression and metastasis (Yachida *et al.* 2010). These more aggressive subclones could gradually replace the less aggressive clones. A potential driver mutation for TCV PTC might be the *BRAF V600E* mutation. Indeed, this work confirms

the fact that *BRAF* mutations are more frequently observed in TCVs as compared to PTCs (Nikiforova *et al.* 2003). Furthermore, it demonstrates that PTCs with *BRAF* mutations have a higher frequency of extrathyroidal extension and nodal metastases than do those that are negative for this mutation (Nikiforova *et al.* 2003).

Although this study was not designed to analyze genetic intratumoral heterogeneity, we observed 11 of 125 tumors with different *BRAF* mutation results in two regions, and the immunohistochemical *BRAF* V600E detection was heterogeneous in two cases, whereas the allelic fraction by pyrosequencing was more than 30% in both cases. These findings support the fact that *BRAF* tumor heterogeneity occurs in PTC (Guerra *et al.* 2012a), but its prognostic role is unclear, because the allelic fraction did not correlate with patient outcome, which is in contrast to previous findings (Guerra *et al.* 2012b). In fact, we did not detect any significant association between the *BRAF* status of the tumors and patient outcome, either when analyzing all of the tumors or when analyzing only the TCV tumors. These findings are supported by other groups that were also not able to find such a relationship (Ito *et al.* 2009, Eloy *et al.* 2011, Koperek *et al.* 2012, Sancisi *et al.* 2012, Barbaro *et al.* 2014). Epidemiologically, it is not surprising that the *BRAF* mutation does not predict ACO, given that *BRAF* mutations can be found in about 40–50% of PTCs, whereas <5% of these tumors have an aggressive clinical course. Therefore, we believe that additional factors have to be present that account for the ACO in patients that harbor this mutation.

One of these potential additional factors could be *TERT* promoter mutations. They were recently discovered to be a novel mechanism of telomerase activation, which is known to be centrally involved in the tumorigenesis of various human cancer types, including thyroid cancer (Vinagre *et al.* 2013). From a diagnostic standpoint, it is worthwhile to mention that *TERT* promoter mutations were not detected in medullary thyroid carcinomas nor in benign lesions or normal thyroids (Vinagre *et al.* 2013). More importantly, recent studies that included TCGA data have reported that *TERT* promoter mutations play a role in aggressive thyroid cancers and in tumor progression from well-differentiated to poorly differentiated and anaplastic thyroid carcinomas. The various studies found a frequency in PTC that ranged from 8 to 25% and was associated with an unfavorable prognosis (Liu *et al.* 2013a,b, Vinagre *et al.* 2013, Cancer Genome Atlas Research 2014, Melo *et al.* 2014, Shimamura *et al.* 2014). They occur significantly more often in TCVs (30%) as compared to conventional PTCs or FVPTCs (10%) and in poorly differentiated thyroid

carcinomas (21–37%) as compared to the differentiated subtypes (PTC or FTC: 12%), and they have a high incidence in anaplastic thyroid carcinomas (13–46%) (Liu *et al.* 2013b, Vinagre *et al.* 2013). Although *TERT* promoter mutations significantly contribute to an ACO in PTC patients, many TCV PTCs with an ACO were *TERT* WT. Thus, other molecular players that are responsible for TC morphology and an ACO have yet to be discovered.

Conclusion

We showed that the presence of as few as 10% TCs within a PTC significantly affects patient prognosis. Therefore, these tumors should be classified accordingly and reported by pathologists, so that treating clinicians are aware that they are dealing with aggressive neoplasms. *BRAF* mutation did not affect patient outcome. Nevertheless, a mutation analysis for *BRAF* might be helpful in clinical management, because a positive *BRAF* status may trigger the search for TCs in order to predict patient outcome, and new therapeutics that inhibit the mutated protein are now clinically available. *TERT* promoter mutations are too rare to serve as a surrogate marker for TCs. However, they are a novel tool for predicting molecularly an adverse outcome in a subset of patients, and their implementation in daily molecular diagnostics is worthwhile.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0057>.

Declaration of interest

D Capper declares shared inventorship of the VE1 antibody and has applied for a patent for the diagnostic use of VE1. Under a licensing agreement between Ventana Medical Systems, Inc., Tucson, AZ, and the German Cancer Research Center, D Capper is entitled to a share of the royalties received by the German Cancer Research Center on the sales of VE1 antibody. The terms of this arrangement are being managed by the German Cancer Research Center in accordance with its conflict of interest policies.

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