TERT, *BRAF*, and *NRAS* in Primary Thyroid Cancer and Metastatic Disease

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Context: Little is known about the frequency of key mutations in thyroid cancer metastases and its relationship with the primary tumor genotype.

Objectives: To evaluate the frequency of *TERT* promoter (*TERT*p), *BRAF*, and *NRAS* mutations in metastatic thyroid carcinomas, analyzing primary thyroid tumors, lymph node metastases (LNMs), and distant metastases.

Design and Patients: Mutation analysis was performed in 437 tissue samples from 204 patients, mainly with papillary thyroid carcinomas (PTCs; n = 180), including 196 LNMs and 56 distant metastases. All the distant metastases included corresponded to radioiodine-refractory metastatic tissue.

Results: We found the following mutation frequency in primary PTCs, LNMs, and distant metastases, respectively: *TERTp*: 12.9%, 10.5%, and 52.4%; *BRAF*: 44.6%, 41.7%, and 23.8%; and *NRAS*: 1.2%, 1.3%, and 14.3%. There was a significant concordance between the primary tumor genotype and the corresponding LNM for all the genes, in particular *BRAF*-mutated PTC. The overall concordance between primary tumors and respective distant metastases was low. In the group of patients with PTCs, we found a high frequency of *TERT*p mutations and a low frequency of *BRAF* mutations in distant metastases, in comparison with the paired primary tumors. When present in distant metastases, *BRAF* mutations frequently coexisted with *TERT*p mutations.

Conclusions: When the genotype of primary tumors is compared with the genotype of LNMs, the concordance is high for all the genes studied. On the other hand, distant metastases show an enrichment in *TERT*p mutations and a decrease in *BRAF* mutations. *TERT*p mutations may play a role in distant metastases. (***J Clin Endocrinol Metab*** **102: 1898–1907, 2017)**

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Abbreviations: ATC, anaplastic thyroid carcinoma; DTC, differentiated thyroid carcinoma; FCDTC, follicular cell–derived thyroid carcinoma; FTC, follicular thyroid carcinoma; LNM, lymph node metastases; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; RAI, radioiodine; TKI, tyrosine kinase inhibitor; *wt*, wild type.

Follicular cell–derived thyroid carcinomas (FCDTCs) are the most frequent endocrine neoplasia (1). The good prognosis of most patients with differentiated FCDTCs—papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC)—relies not only on their slow growth, but also on the success of radioiodine (RAI) as a therapeutic strategy. Although patients with PTC, by far the most frequent histotype of FCDTCs, have a low mortality rate, about 20% have persistent or recurrent local disease during follow-up (2, 3). Among the different clinical, biochemical, and imaging prognostic factors that have been advanced (4), the presence of clinically evident neck lymph node metastases (LNM) has been shown to be a predictor of persistent or recurrent disease during follow-up (5). On the other hand, the existence of distant metastases is one of the most powerful predictors of poor outcome, including disease-specific mortality, particularly in the absence of RAI uptake from metastatic tissue (6, 7).

Over the last years, several genetic alterations have been identified in FCDTCs, which mainly involve genes of the MAP kinase pathway (*RET/PTC* rearrangements, *BRAF* and *RAS* point mutations). The V600E mutation represents the vast majority of *BRAF* mutations, whereas mutations in *NRAS* codon 61 (Q61R or Q61K) are the most prevalent among *RAS* gene family mutations (8, 9). *TERT* promoter mutations (*TERT*p) were recently described as important events in FCDTCs, being present in a significant proportion of cases (10–12). The value of *BRAF*, *TERT*p, and *RAS* mutations as prognostic biomarkers has been assessed in a number of studies (13). *BRAF* mutations seem to be associated with markers of clinical aggressiveness (larger tumors, older age, extrathyroidal extension, and LNM) and poor clinical outcome, although the latter association appears to be dependent on additional clinico-pathological features (14–18). *BRAF* mutations have also been associated with decreased expression of the sodium-iodine symporter, a crucial factor determining response to RAI therapy (19–21). On the other hand, *BRAF* mutations have not been associated with distant metastases in the majority of studies (18, 22–25); indeed, patients with *BRAF*-mutated tumors may develop distant metastases less frequently than patients with *BRAF* wild-type (*wt*) tumors (26), emphasizing how challenging the use of *BRAF* mutations as a prognostic marker can be in clinical practice. The prognostic role of *RAS* mutations is not established yet, but their presence has been associated with distant metastases (27). Although less data are currently available about the clinical significance of *TERT*p mutations, all the published series, including ours (28), reported an association between the presence of such mutations and clinically aggressive disease, including RAI refractoriness (11, 12, 22, 23, 28–31). In accordance with these data, clinical guidelines recently incorporated the genotype of *BRAF* and *TERT*p in the prognostic stratification criteria (3).

Although there are abundant data regarding the frequency of *BRAF*, *TERT*p, and *RAS* mutations in thyroid tumors and their association with clinico-pathological features, response to therapy, and outcome, little is known about the frequency of such mutations in metastases (30, 32), particularly in distant metastases. This is especially relevant, because clinically evident local metastases are known to be predictors of disease persistence/recurrence, and distant metastases are an important indicator of poor prognosis (3). Further information about the metastases' mutational status and its relationship with the primary tumor genotype is therefore a burning need. In addition, in the era of personalized medicine and systemic targeted therapies for advanced disease, a comprehensive understanding of the molecular alterations present in metastatic tissue is crucial to assess the relationship between response to therapy and tumor tissue genotype.

In the current study, we searched for the presence of *TERT*p, *BRAF*, and *NRAS* mutations in a large series of thyroid tumors, LNM, and RAI-refractory distant metastases and investigated the concordance of the mutational status between the primary tumor and corresponding metastases.

Materials and Methods

All the procedures described in this study were in accordance with national and institutional ethical standards. Patients signed an informed consent form approved by the internal reviewing board.

Patient tissue samples

Four hundred thirty-seven formalin-fixed, paraffin-embedded tissue samples from thyroid tumors, LNM, and distant metastases were collected from the files of the Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal, corresponding to 204 patients treated and followed in five university hospitals in Portugal and Spain (Table 1). Because none of the institutions performed routine prophylactic lymph node dissection, all the lymph nodes analyzed corresponded to clinically evident LNM or lymph nodes resected during thyroidectomy based on their suspicious appearance. All the distant metastases analyzed in the current study corresponded to RAI-refractory metastatic tissue. All the metastatic tissue was retrieved from surgical specimens.

The histology of all tumor samples (180 PTC, 15 FTC, six poorly differentiated thyroid carcinomas (PDTCs], and one anaplastic thyroid carcinoma [ATC]) was revised by three pathologists (M.J.M., J.M.C.-T., and M.S.-S.) in accordance with the World Health Organization criteria (1). In two patients, from whom only metastatic tissue was available, it was not possible to retrieve accurate information about the histologic subgroup of the primary tumor. Data on the clinico-pathological characteristics of patients and tumors are summarized in Table 2.

Genetic characterization of tumors

DNA extraction

DNA from formalin-fixed, paraffin-embedded tissues was retrieved from 10-μm sections after careful microdissection. In

Table 1. Number of Patients Included in the Study and Number of Tumor Specimens Analyzed (Thyroid Tumors, LNM, and Distant Metastases)

	n
Number of patients included in the study	204
Number of patients with thyroid tumors analyzed	185
Number of patients with LNM analyzed	164
Total number of LNM analyzed[a]	196
Number of patients with distant metastases analyzed	42
Total number of distant metastases analyzed[b]	56
Patients with pairing of thyroid tumor and LNM	159
Patients with pairing of thyroid tumor and distant metastases	27

[a]In 18 patients, specimens from two LNMs were analyzed; in seven patients, specimens from three LNM were analyzed.

[b]In five patients, specimens from two distant metastases were analyzed; in three patients, specimens from three distant metastases were analyzed; and in one patient, specimens from four distant metastases were analyzed.

23 large tumors displaying areas with different histological pattern, DNA was extracted from different areas after separate microdissection. DNA extraction was performed using the Ultraprep Tissue DNA Kit (AHN Biotechnologie, Nordhausen, Germany) following the manufacturer's instructions.

PCR and Sanger sequencing

The genetic characterization of *TERT*p, *BRAF*, and *NRAS* genes was performed in thyroid carcinomas, LNM, and distant metastases as previously reported (28, 33). All the detected mutations were further validated by a new independent analysis in both strands. In the 23 thyroid tumors with multiple samples from areas showing different architecture, we considered the tumor as mutated for a particular gene if at least one of the samples harbored a mutation.

Statistical analysis

Statistical analysis was conducted with SPSS version 20.0 (SPSS Inc., Chicago, IL). The results are expressed as percentage or mean ± standard deviation. Statistical analysis was performed both in the whole series of FCDTCs and in the different tumor groups. The results obtained in patients with PDTCs and ATCs were grouped together to increase the statistical power. A Kruskal-wallis test, χ^2 test, Fisher's exact test, *t* test (unpaired, two-tailed), and analysis of covariance were used whenever appropriate. Cohen's kappa statistics was used to evaluate genotype concordance between primary thyroid tumors and

Table 2. Epidemiological, Histological, and Clinical Data of Patients With FCDTCs Included in the Study

	Total[a]	PTCs	FTCs	PDTCs + ATCs
Total number	204	180	15	7
Age at diagnosis (n)	195	174	14	6
Mean (years)	46.8 ± 16.6	45.3 ± 16.5	55.6 ± 13.3	54.7 ± 10.2
<45 years	96 (49.2)	94 (54.0)	2 (14.3)	0 (0.0)
≥45 years	99 (50.8)	80 (46.0)	12 (85.7)	6 (100.0)
Sex (n)	196	174	14	6
Female	136 (69.4)	125 (71.8)	9 (64.3)	2 (33.3)
Male	60 (30.6)	49 (28.2)	5 (35.7)	4 (66.7)
Tumor size (n)	179	167	8	4
<2 cm	104 (58.1)	102 (61.1)	2 (25.0)	0 (0.0)
2–4 cm	49 (27.4)	46 (27.5)	3 (37.5)	0 (0.0)
>4 cm	26 (14.5)	19 (11.4)	3 (37.5)	4 (100.0)
Extrathyroidal extension (n)	183	168	11	4
Present	133 (72.7)	128 (76.2)	2 (18.2)	3 (75.0)
Vascular invasion (n)	109	99	6	4
Present	51 (46.8)	41 (41.4)	6 (100.0)	4 (100.0)
Lymph node metastasis (n)	194	173	14	6
Present	170 (87.6)	165 (95.4)	3 (21.4)	1 (16.7)
Distant metastasis (n)	204	180	15	7
Present	57 (27.9)	35 (19.4)	14 (93.3)	7 (100.0)
Stage (sixth UICC/AJCC) (n)	186	169	12	5
I	89 (47.9)	87 (51.5)	2 (16.7)	0 (0.0)
II	6 (3.2)	5 (3.0)	1 (8.3)	0 (0.0)
III	66 (35.5)	63 (37.2)	1 (8.3)	2 (40.0)
IV	25 (13.4)	14 (8.3)	8 (66.7)	3 (60.0)
TERT promoter (n)	182	163	12	6
wt	153 (84.1)	142 (87.1)	6 (50.0)	4 (66.7)
Mutation	29 (15.9)	21 (12.9)	6 (50.0)	2 (33.3)
BRAF (n)	185	166	12	6
wt	110 (59.5)	92 (55.4)	12 (100.0)	5 (83.3)
Mutation	75 (40.5)	74 (44.6)	0	1 (16.7)

n = number of patients with available data for each feature. Numbers between parentheses represent percentages within each category.

Abbreviations: AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control.

[a]In two patients, from whom only metastatic tissue was available, it was not possible to retrieve information about the histologic subgroup of the primary tumor.

Table 3. Frequency of *TERT*p, *BRAF*, and *NRAS* Mutations in Primary Thyroid Tumors, LNM, and Distant Metastases According to the Different Histotypes

		Thyroid Tumors	LNM	Distant Metastases
FCDTCs		n = 185	n = 164	n = 42
*TERT*p	mut	29/182 (15.9)	17/157 (10.8)	22/42 (52.4)
BRAF	mut	75/185 (40.5)	65/160 (40.6)	7/42 (16.7)
NRAS	mut	3/181 (1.7)	2/159 (1.3)	5/42 (11.9)
DTCs		n = 178	n = 162	n = 34
*TERT*p	mut	27/175 (15.4)	16/155 (10.3)	17/34 (50.0)
BRAF	mut	74/178 (41.6)	65/158 (41.1)	6/34 (17.6)
NRAS	mut	3/174 (1.7)	2/157 (1.3)	5/34 (14.7)
PTCs		n = 166	n = 160	n = 21
*TERT*p	mut	21/163 (12.9)	16/153 (10.5)	11/21 (52.4)
BRAF	mut	74/166 (44.6)	65/156 (41.7)	5/21 (23.8)
NRAS	mut	2/162 (1.2)	2/155 (1.3)	3/21 (14.3)
FTCs		n = 12	n = 2	n = 13
*TERT*p	mut	6/12 (50.0)	0/2 (0.0)	6/13 (46.2)
BRAF	mut	0/12 (0.0)	0/2 (0.0)	1/13 (7.7)
NRAS	mut	1/12 (8.3)	0/2 (0.0)	2/13 (15.4)
PDTCs + ATCs		n = 6	n = 1	n = 7
*TERT*p	mut	2/6 (33.3)	1/1 (100.0)	4/7 (57.1)
BRAF	mut	1/6 (16.7)	0/1 (0.0)	1/7 (14.3)
NRAS	mut	0/6 (0.0)	0/1 (0.0)	0/7 (0.0)

corresponding LNM and distant metastases. For pairing analysis with primary tumors, patients with more than one lymph node metastasis or distant metastases were considered to have mutated metastases if a mutation was found in at least one of them.

Differences were considered statistically significant whenever $P < 0.05$.

Results

From the 437 tissue samples (204 patients) included in the study, primary tumor tissue was available for genetic analysis in 185 patients. One hundred ninety-six LNM belonging to 164 patients and 56 distant metastases from 42 patients were also analyzed (Table 1). The histotypes of the primary tumors corresponding to the distant metastases included in our study were as follows: 21 PTCs, 13 FTCs, and 7 PDTCs; in one patient, only metastatic tissue was available, and it was not possible to retrieve accurate information about the primary tumor.

The complete description of the series, with clinicopathological data, is displayed in Table 2.

Frequency of TERTp mutations in thyroid tumors, LNM, and distant metastases

In the whole FCDTC series, *TERT*p mutations were present in 15.9% of cases (Table 3; Fig. 1). Within differentiated thyroid carcinomas (DTCs) *TERT*p mutations were detected in 15.4% of cases (12.9% in PTC and 50.0% in FTC), whereas in PDTCs/ATCs, there were 33.3% of *TERT*p-mutated cases.

The association of *TERT*p mutations with clinicopathological characteristics is summarized in Supplemental Table 1. Briefly, in the group of patients with DTCs, *TERT*p mutations were associated with older age at diagnosis, larger tumors, presence of distant metastases, and higher tumor stage.

*TERT*p mutations were found in 10.8% of LNM. The frequency of *TERT*p mutations in LNM according to the histotype of the primary tumor was as follows: 10.3% of DTCs, 10.5% of PTCs, and 0% of FTCs. A *TERT*p mutation was detected in the only LNM from a patient with PDTC, which was available for analysis.

Considering the entire series, *TERT*p mutations were found in 52.4% of distant metastases, evenly distributed among the distant metastases from the different primary tumor histotypes: 50.0% in metastases from DTCs (52.4% from PTCs and 46.2% from FTCs) and 57.1% in metastases from PDTCs/ATCs. *TERT*p mutations were found in 36.4% of lung, 50.0% of bone, 66.7% of skin, and 100% of brain metastases (Supplemental Table 2).

In addition to the most common -124G>A and -146G>A mutations, three other genetic alterations involving the promoter region of *TERT* were detected (Supplemental Figs. 1 and 2). Of note, the most frequent mutation in thyroid tumors and LNM was the -124G>A alteration (61.3% and 70.6%, respectively), whereas in distant metastases, the predominant mutation was the -146G>A (48.2%) (Supplemental Fig. 2). Complex alterations involving *TERT*p were also more frequently found in distant metastases. Six cases showed heterogeneity

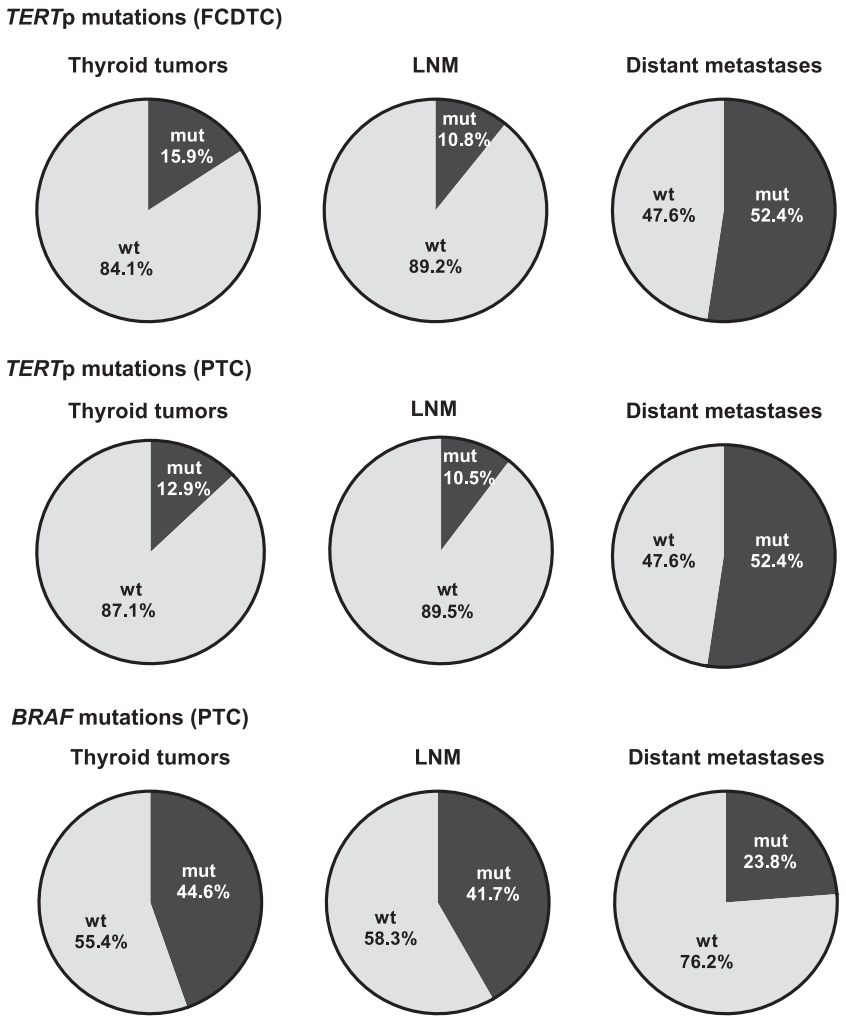

Figure 1. Frequency of *TERT*p and *BRAF* mutations in primary thyroid tumors, LNM, and distant metastases.

regarding *TERT*p genotype in primary tumor and metastatic tissue (Supplemental Table 3).

Frequency of BRAF mutations in thyroid tumors, LNM, and distant metastases

Taking into consideration that *BRAF* mutations are characteristic of PTCs and are also found in PDTCs/ATCs, the results are presented in these two groups.

Confirming their close relationship, *BRAF* mutations were detected in 44.6% of PTCs, whereas no mutations were found in FTCs. *BRAF* mutations were also present in 41.7% of LNM and in 23.8% of distant metastases from PTC cases (Fig. 1). Furthermore, there was a significant association between the presence of *BRAF* mutations in primary tumors and occurrence of LNM ($P = 0.03$). The association of *BRAF* mutations with clinicopathological characteristics is summarized in Supplemental Table 1.

From the six PDTC/ATC cases analyzed for *BRAF*, only one (16.7%) harbored a mutation, which was also detected in the respective distant metastasis (14.3% of all distant metastases). No *BRAF* mutations were detected in the remaining distant metastases, nor in the single lymph node metastasis available from this group of patients.

Considering that *TERTp*-mutated tumors frequently also harbor *BRAF* mutations, we analyzed the coexistence of both mutations in primary PTCs, LNM and distant metastases (Table 4). *TERTp*-only mutations were present in 7.3%, 5.3%, and 33.3% of thyroid tumors, LNM, and distant metastases, respectively. BRAF-only mutations were present in 39.3%, 35.8%, and 4.8% of thyroid tumors, LNM, and distant metastases, respectively. Simultaneous *TERT*p and *BRAF* mutations were present in 5.5%, 5.3%, and 19.0% of thyroid tumors, LNM, and distant metastases, respectively.

Frequency of NRAS mutations in thyroid tumors, LNM, and distant metastases

In the whole FCDTC series, *NRAS* mutations were detected in 1.7% of cases, corresponding to two cases of PTC and one case of FTC, all harboring the Q61R mutation. The frequency was similar in LNM (1.3%; two patients with the Q61R mutation) but slightly higher in distant metastases (11.9%; four patients with the Q61R mutation and one with the Q61K mutation).

Pairing of thyroid tumor genotype for TERTp and BRAF with the genotype from LNM and distant metastases

Whenever the data were available, we performed a paired analysis of the genotype of the primary tumor, LNM, and distant metastases.

Regarding *TERT*p genotype (*wt vs* mutated), the overall concordance between primary tumors and LNM was 88.0% ($\kappa = 0.33$; $P < 0.001$) (Tables 5–7): in particular, 94.0% of *TERT*p *wt* tumors were paired with *wt* LNM, whereas 37.5% *TERTp*-mutated tumors were concordant with the LNM. The overall concordance between primary tumors and distant metastases was 69.2% ($\kappa = 0.37$; $P = 0.059$). More specifically, 73.3% of *TERT*p *wt* tumors were paired with *wt* distant metastases, whereas 63.6% of *TERTp*-mutated tumors were paired with mutated distant metastases.

Table 4. Frequencies of Combined *TERT*p and/or *BRAF* Mutations in Primary Papillary Thyroid Carcinomas, LNM, and Distant Metastases

Genotype	Thyroid Tumor (N = 163): n (%)	LNM (N = 151): n (%)	Distant Metastases (N = 21): n (%)
*TERT*p_*wt*/*BRAF_wt*	78 (47.9)	81 (53.6)	9 (42.9)
*TERT*p_*wt*/*BRAF*_Mut	64 (39.3)	54 (35.8)	1 (4.8)
*TERT*p_Mut/*BRAF_wt*	12 (7.3)	8 (5.3)	7 (33.3)
*TERT*p_Mut/*BRAF*_Mut	9 (5.5)	8 (5.3)	4 (19.0)

For BRAF, the overall concordance between the genotype of primary tumors and LNM was 69.0% (κ = 0.37; $P < 0.001$); 75.6% of BRAF wt thyroid tumors were matched with wt LNM, whereas 60.9% of BRAF-mutated tumors were paired with mutated LNM. The overall concordance between thyroid tumors and distant metastases was 77.8% (κ = 0.13; P = 0.484); 83.3% of BRAF-wt thyroid tumors were matched with wt distant metastases, whereas 33.3% of BRAF-mutated thyroid tumors were paired with mutated distant metastases.

All the patients with *NRAS wt* primary tumors also tested negative in the LNM. The two patients with *NRAS*-mutated primary tumors and paired metastatic tissue available for analysis showed the same mutation in the LNM (overall concordance of 100% [κ = 1.00; $P < 0.001$]). Only one of the two had distant metastases harboring this mutation. The overall concordance primary tumor/distant metastases was 96.3% (κ = 0.65; $P < 0.001$).

Discussion

The precise molecular stratification of tumors is crucial for an effective targeted therapy and for translating personalized medicine from concept to routine clinical practice. In thyroid cancer, two forms of systemic targeted therapy exist: RAI and tyrosine kinase inhibitors (TKIs); therefore, it is crucial to understand the relationship between molecular alterations and key factors determining response to both therapies.

In this study, we evaluated the mutational status of *TERT*p, *BRAF*, and *NRAS* in FCDTCs and their regional (LNM) and distant metastases. Although the overall frequency of *TERT*p and *BRAF* mutations was similar in primary tumors and in LNM, distant metastases showed a marked increase in *TERT*p, together with a decrease in *BRAF* mutation frequency.

In patients with FCDTCs, the natural history and clinical implications of local and distant metastases seem to be very distinct. LNM are often present at diagnosis and are the most frequent cause of local recurrences in PTCs; nonetheless, the prognostic significance of LNM is dependent upon several characteristics, like size, number, and presence of extranodal extension (34). On the other hand, only 50% of distant metastases are present at diagnosis, and they are crucial determinants of patients' outcome, being associated with increased disease-specific mortality, especially when they are RAI refractory (6, 7).

The concordance of the genotype between primary tumors and LNM is high. This finding reinforces the concept that the process of local metastasis does not implicate the acquisition of new molecular alterations (35). It also means that *BRAF* may play a role in the frequent local metastasis process within the setting of PTCs. Nevertheless, our previous studies had shown that LNM were more closely related with the infiltrative growth pattern, invasive features, and intratumoral lymph vessel density, rather than with the presence of *BRAF* or *RAS* mutations (36, 37). Furthermore, chemokines like CXCL8 or CXCR4 were found to play a role in the invasiveness and progression of several tumors, including FCDTCs (38), and the density of tumor-associated macrophages was associated with the presence of LNM, regardless of the *BRAF* status in PTCs (39).

Table 5. Concordance of Genotype (*TERT*p) Between Primary Thyroid Tumors, LNM, and Distant Metastases

		*TERT*p in Thyroid Tumors			
	Total	Wild Type	Mutated	κ	*P* Value
*TERT*p in LNM					
Wild type	136	126 (84.0)	10 (6.7)	0.334	<0.001
Mutated	14	8 (5.3)	6 (4.0)		
*TERT*p in distant metastases					
Wild type	15	11 (42.3)	4 (15.4)	0.370	NS (0.059)
Mutated	13	4 (15.4)	7 (26.9)		

Abbreviation: NS, Not significant.

Table 6. Concordance of Genotype (*BRAF*) Between Primary Thyroid Tumors, LNM, and Distant Metastases

		BRAF in Thyroid Tumors			
	Total	Wild Type	Mutated	κ	*P* Value
BRAF in LNM					
Wild type	92	65 (41.9)	27 (17.4)	0.368	<0.001
Mutated	63	21 (13.5)	42 (27.1)		
BRAF in distant metastases					
Wild type	22	20 (74.1)	2 (7.4)	0.129	NS (0.484)
Mutated	5	4 (14.8)	1 (3.7)		

Abbreviation: NS, Not significant.

Taking all these data into account, we can hypothesize that the process of nodal metastasis appears to be more dependent upon the morphologic characteristics of the tumor and its microenvironment elements than on specific molecular alterations.

The current study shows that, in contrast to the setting of LNM, differences emerge when comparing primary tumors and distant metastases. Although the frequency of *TERT*p mutations is higher in distant metastases (52.4%) than in primary thyroid tumors (15.9%), the opposite happens for *BRAF* mutations in PTCs, which are more frequently detected in primary tumors (44.6%) than in distant metastases (23.8%). If we consider the simultaneous occurrence of *TERT*p and *BRAF* mutations or the isolated presence of one of these mutations in PTCs (Table 4), the same trend occurs: although *BRAF*-only mutations are the most frequent molecular alterations in LNM, *TERTp*-only mutations are the most frequent alteration in distant metastases. Of note, when present in distant metastases, *BRAF* mutations coexist with *TERT*p mutations, being that the isolated presence of *BRAF* mutations in distant metastases is notably rare (4.8%). Our results reinforce the strong association, previously advanced by our group (28) and others (40–42), between *TERT*p mutations in thyroid tumors and the presence of distant metastases. Our findings also confirm, now with evidence obtained from metastatic tissue, the results by Sancisi *et al.* (26), who reported less-frequent distant dissemination in PTCs harboring *BRAF* mutations. Using an approach similar to ours, Ricarte-Filho *et al.* (32) reported a high frequency of *BRAF* mutations in a smaller series of advanced primary and metastatic RAI-refractory thyroid cancers, where metastatic tissue was available from 12 patients (19 distant metastases). The difference between our results and those of Ricarte-Filho *et al.* may reside in our set of patients, which comprised a higher proportion of patients with DTCs and a greater number of distant metastases (n = 56), thus providing more robust results about the molecular profile of distant metastases in FCDTCs. Furthermore, the study by Ricarte-Filho *et al.* also included a great proportion of patients with the tall-cell variant of PTCs and PDTCs, and this may be one of the reasons why the frequency of *BRAF* mutations is higher in their study.

In distant metastases, *TERT*p mutations were more frequent in bone and brain than in lung metastases (Supplemental Table 2). We do not have a definitive explanation for this, but we reason that it may be due to the biological similarities between lung and thyroid: being both epithelial tissues, commonly expressing transcription factors like TTF-1, it is tempting to assume that homing might be easier in lung than in bone or brain; in the latter, cells may face more adverse conditions, rendering them more dependent on important survival factors like *TERT*p mutations.

In our study we only analyzed the most frequently mutated gene of the *RAS* gene family, *NRAS*, which represents more than two-thirds of all mutations involving the *RAS* gene family in FCDTCs. The frequency of *NRAS* mutations was lower than previously reported,

Table 7. Concordance of Genotype (*NRAS*) Between Primary Thyroid Tumors, LNM, and Distant Metastases

		NRAS in Thyroid Tumors			
	Total	Wild Type	Mutated	κ	*P* Value
NRAS in LNM					
Wild type	149	149 (98.7)	0	1.000	<0.001
Mutated	2	0	2 (1.3)		
NRAS in distant metastases					
Wild type	25	25 (92.6)	0	0.649	<0.001
Mutated	2	1 (3.7)	1 (3.7)		

especially in FTCs (8, 9), a finding that may, in part, be explained by the small number of patients with FTCs from whom primary thyroid tumors were available. Nevertheless, we observed an increase in the *NRAS* mutation frequency in distant metastases. Because *NRAS* mutations in thyroid tumors were previously associated with increased risk of distant metastasis (27), our data support the assumption that this molecular alteration may play a role in the dissemination process.

The identification of plasma biomarkers as a tool in the follow-up of patients with cancer is a very interesting topic. In thyroid cancer, the detection of *BRAF* mutations in cell-free DNA in the plasma of patients with PTCs is feasible (43) and may help in the diagnosis (44), and there are preliminary data showing that this method may also be useful in the follow-up of patients through the detection of *BRAF* mutation in the plasma of patients with persistent disease (43). The results of our study raise some doubts about the practical usefulness of such methods in the identification of patients with distant metastases from PTCs. The fact that distant metastases from PTCs show a decrease in the frequency of *BRAF* mutations may decrease the sensitivity of cell-free DNA analysis in this setting. Further studies are needed to specifically address this issue.

Thyroid cancer has been described as a tumor type in which metastatic dormancy seems to be a frequent phenomenon, because biochemical disease may be detectable years before metastatic disease or progression can be conclusively detected by imaging (45). Tumor heterogeneity is likely also an issue to consider in studies comparing specific molecular alterations in primary tumors and matching metastases. In fact, whenever discrepancy between primary tumors and metastases is found, two major explanations emerge: a new molecular alteration took place at the metastatic site or a subclone of the primary tumor with increased ability to metastasize actually succeeded and gave rise to metastatic tissue. In this context, it seems that *BRAF* does not provide an advantage for distant metastasis, despite being associated with invasiveness (intravasation); this fact may be related to decreased survival capabilities in the blood, less efficient extravasation, or less efficient homing at the distant sites. On the other hand, the enrichment of metastatic tissue with *TERT*p mutations also raises important questions. We can hypothesize that either (1) a small pool of *TERT*p-mutated circulating tumor cells may have increased survival abilities in the circulation/homing phase or (2) the gain of *TERT*p mutations at the secondary location may be a fundamental step toward the development of clinically evident metastases. Either way, our data reinforce the concept that *TERT*p mutations are a key event in the process of distant dissemination.

As long as there is evidence of RAI uptake, RAI remains the cornerstone of metastatic FCDTC treatment (3). In this setting, surgery is usually reserved for RAI-refractory lesions. In our series, we only included surgical specimens of metastatic tissue, minimizing possible sampling bias that may occur when samples from biopsies are analyzed for molecular alterations, as the genotype of the biopsy specimen may not be representative of the entire lesion. On the other hand, a possible limitation of our work is the fact that we have only studied RAI-refractory distant metastases, and therefore our series may not reflect the frequency of molecular alterations found in more frequent RAI-avid/responsive distant metastases. In a previous study, we observed that patients with tumors harboring *TERT*p mutations had been submitted to a significantly higher number of RAI treatments, with higher cumulative doses (28); another group recently found an association between *TERT*p mutations and non–RAI-avid DTCs (31). These findings raised the hypothesis that *TERT*p mutations could be associated with RAI resistance, as previously advanced for *BRAF*-mutated tumors (19, 21). *TERT*p and *BRAF* mutations may be factors to consider when individualizing RAI doses.

With the emergence of personalized medicine and systemic targeted therapies to treat RAI-resistant and progressive metastatic disease, a deeper knowledge of the metastatic tissue is vital to evaluate if and how the metastasis-associated genetic alterations may modulate therapy response. Until now, the trials evaluating TKI in the treatment of RAI-refractory disease have only considered the genotype of the primary tumor in subgroup analysis (46, 47), whereas the real frequency of the molecular alterations in metastatic tissue and its relationship with the genotype of primary thyroid tumors remain largely unknown. Our results demonstrate that, in a considerable proportion of cases, the genotype of persistent/recurrent disease is different from that of the primary tumors, a finding that may provide an explanation for the lack of association between tumor genotype and therapy response. We believe that the design of future trials should take into account the genotype of metastatic tissue, especially when considering the use of specific inhibitors, like vemurafenib for *BRAF*-mutated tumors.

We conclude that regional and distant metastases of FCDTCs show a different molecular profile: whereas LNM display high concordance of genotypes with primary thyroid tumors, distant metastases show a different pattern, with a marked enrichment in *TERT*p and a reduction of *BRAF* mutations. These findings are of the upmost importance when considering the use of TKIs in the treatment of advanced metastatic thyroid cancer. *TERT*p mutations may play a role in the development of RAI-refractory distant metastases.

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