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PROGNOSTIC SIGNIFICANCE OF *TERT* PROMOTER AND *BRAF* MUTATIONS IN TIR-4 AND TIR-5 THYROID CYTOLOGY

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ABSTRACT

Objective: Follicular-derived thyroid cancers generally have a good prognosis, but in a minority of cases they have an aggressive behavior and develop distant metastases, with an increase in the associated mortality. None of the prognostic markers currently available prior to surgery can identify such cases. **Methods:** *TERT* promoter and *BRAF* gene mutations were examined in a series of 436 consecutive TIR-4 and TIR-5 nodes referred for surgery. Follow-up (median: 59 months, range: 7-293 months) was available for 384/423 patients with malignant nodes. **Results:** *TERT* promoter and *BRAF* mutations were detected in 20/436 (4.6%), and 257/434 thyroid nodules (59.2%), respectively. At the end of the follow-up, 318/384 patients (82.8%) had an excellent outcome, 48/384 (12.5%) had indeterminate response or biochemical persistence, 18/384 (4.7%) had a structural persistence or died from thyroid cancer. *TERT* promoter mutations correlated with older age ($p<0.0001$), larger tumor size ($p=0.0002$), oxyntic and aggressive PTC variants ($p=0.01$), higher tumor stages ($p<0.0001$), distant metastases (<0.0001) and disease outcome ($p<0.0001$). At multivariate analysis, *TERT* promoter mutation was not an independent predictor of disease outcome. *TERT* promoter mutations (OR 40.58; 95% CI 3.06 to 539.04), and N1b lymph node metastases (OR 40.16, 95% CI 3.48 to 463.04) were independent predictors of distant metastases. *BRAF* mutation did not predict the outcome, and it correlated with a lower incidence of distant metastases ($p=0.0201$). **Conclusions:** *TERT* promoter mutation proved an independent predictor of distant metastases, giving clinicians the chance to identify many of the patients who warranted more aggressive initial treatment and closer follow-up.

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INTRODUCTION

Follicular-derived thyroid cancer (FDTC) is the most common endocrine cancer (1), and it is being diagnosed more and more frequently, possibly due to the increasing use of neck ultrasound for thyroid diseases and other unrelated conditions (2). The prognosis for FDTC is usually good, the 10-year survival rate ranging between 85% and 93% (1). There is a tendency to treat it using less invasive surgical procedures, and less radioiodine, with a weaker ^{131}I activity (3). From 7% to 20% of FDTC recur or persist, however, especially if initial surgery is not radical (3, 4), and patients who require additional treatments and intensive follow-up may experience a higher morbidity and worse quality of life. The 5-year survival rate in cases of poorly-differentiated thyroid carcinoma (PDTC), and in patients with distant metastases is also significantly worse, at 72% and 30-50%, respectively (5). Distant metastases are not very common, occurring in only 2-5% of cases, but they have a significant impact on patient outcome and quality of life (6). Unlike other cancers, in the case of FDTC, metastatic disease is amenable to treatment (providing the primary tumor has been completely removed) because it may respond to RAI therapy (3).

In the light of the above considerations, it is time to adjust surgical and radioiodine strategies to the individual risk of disease persistence or recurrence, distant metastases, and death. This involves identifying the minority of patients with aggressive FDTC who warrant a more aggressive treatment and closer follow-up (2). Unfortunately, good prognostic indicators - available before surgery, or already at the time of a patient's diagnosis - are still lacking.

Fine-needle aspiration (FNA) cytology has an essential role in the diagnosis of thyroid nodules, and the value of the molecular markers obtained thereby has been widely investigated, especially for indeterminate cytologies (3). The significance of BRAF mutation as a prognostic molecular marker has also been investigated in numerous studies (7). According to some reports, BRAF mutations appear to be associated with large tumor size (8, 9), extension beyond the thyroid (8, 9), advanced stage at diagnosis (8, 10), and lymph node involvement (9, 11). How *BRAF* mutations correlate

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with outcome is a more complicated issue, however. A systematic meta-analysis found a significantly higher independent recurrence rate in *BRAF V600E* mutated than in *BRAF* wild-type tumors (11), but it is hard to say whether the BRAF mutation was an independent risk factor, or whether said higher recurrence rate was due to aggressive clinicopathological features associated with the mutational status (3). *BRAF* mutation was found unassociated with any presence of distant metastases in most studies (12, 13, 14). Sancisi *et al* even found that distant metastases developed less frequently in *BRAF*-mutated than in wild-type tumors (15). Melo *et al* confirmed as much from the molecular standpoint, finding *BRAF* mutation less often in distant metastases than in their paired primary tumors (16). Assessing *BRAF* mutation in isolation is therefore not enough for proper risk stratification (3).

Telomerase reactivation or re-expression is a hallmark of cancer and allows unlimited proliferation. Somatic mutations in the promoter region of telomerase reverse transcriptase (TERT) have been found in a large proportion of human tumors (16), including FDTC (17). In particular, the rates of *TERT* promoter mutation in thyroid specimens reportedly range from 7% (18, 19) to 23% (20) in PTC, from 11.4% (17) to 32% (21) in FTC (with higher rates in aggressive cancers), from 29% (19) to 43% (18) in PDTC, and from 33% (19) to 51% in ATC (20). These mutations occur -124 and -146 base-pairs away from the TERT translation start site [1,295,228 C>T (C228T) and 1,295,250 C>T (C250T)] (18). They increase *TERT* promoter activity, giving rise to new consensus sites for transcription factors. The literature has consistently demonstrated an association between *TERT* promoter mutation and older-aged patients, larger tumors, distant metastases, and advanced stage at diagnosis (18, 19). On the other hand, *TERT* mutations do not seem to be associated with lymph node metastases (18, 19, 22), and an association with extrathyroidal extension and vascular invasion has emerged in many, but not all reports (18, 19, 22). More importantly, *TERT* promoter mutations seem to independently predict patient mortality (19), and disease-free survival (18, 19, 23), and this makes them seem promising as a way to identify tumors with more aggressive anatomopathological

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features and a worse prognosis. All the above-mentioned studies analyzed the *TERT* promoter in cancer tissue that became available after surgery, however. Its prognostic value in FNA from thyroid nodes prior to surgery remains unknown as yet, and the question is challenging, partly because *TERT* promoter mutations are often subclonal in thyroid cancer tissue (19). The aim of the present study was thus to elucidate the prognostic value of *TERT* promoter mutations in a large, single-center, consecutive series of cytologically malignant or suspect thyroid nodules.

MATERIAL AND METHODS

The study involved 436 samples found malignant or suspect on FNA cytology, obtained from thyroid nodules in 436 consecutive adult patients referred for surgical excision. Molecular analysis for somatic *TERT* promoter mutations was performed retrospectively in all patients. *BRAF* analysis was performed as well for 434 patients. All studies were conducted in accordance with the guidelines of the Declaration of Helsinki. The present study was approved by our local ethical committee (Azienda Ospedaliera di Padova, approval code number: AOP1303), and all patients gave their written informed consent to the use of their thyroid cytology findings for research purposes. In the present series, the decision regarding the extent of initial surgery, considering total thyroidectomy and prophylactic neck compartment dissection, was based on patients' clinical status, and the surgeons' and patients' preferences: 434/436 patients (99.5%) underwent total thyroidectomy, 1/436 (0.2%) had a lobectomy, and 1 (0.2%) had no surgery due to a diagnosis of anaplastic thyroid carcinoma. All 436 cases collected were classified according to the SIAPEC 2014 consensus statement (24). ^{131}I remnant ablation was performed in 387 patients (median dose: 100 mCi; range: 30-200 mCi). Histological diagnostics and staging were done according to the TNM classification, considering both the 7th (25), and the 8th (26) editions, and on the grounds of the first whole-body scan after ^{131}I remnant ablation. All patients with a negative whole-body scan outside the thyroid bed, negative thyroid ultrasound (US), and undetectable thyroglobulin (Tg) with

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negative thyroglobulin autoantibodies after therapy underwent rhTSH-stimulated Tg assessment 12 months after remnant ablation, according to standard procedures. Then patients were routinely followed up every 6 or 12 months. Additional Tg assays, FNA cytology, or 18F-FDG PET were performed, depending on patients' clinical features, or when persistent disease was suspected. Further ^{131}I and/or surgical treatments, and/or external radiotherapy, and/or tyrosine kinase inhibitor treatment were administered if further disease was confirmed. Patient outcome was classified as a "biochemically incomplete or structurally incomplete response", "indeterminate" or an "excellent response", according to the American Thyroid Association (3) guidelines for patients undergoing remnant ablation, and according to the criteria proposed by Momesso *et al.* (27) for patients not receiving ^{131}I treatment. The median patient follow-up was 59 months (range: 7-293 months); 39/423 (9.2%) patients with malignant disease were lost to follow-up.

***BRAF* and *TERT* mutation analysis**

DNA was isolated from FNA samples using the QIAamp DNA Micro kit (Qiagen, Italy) according to the manufacturer's protocol. *BRAF* (NM_004333.4) exon 15 and *TERT* proximal promoter (NM_198253.2) status was assessed by direct sequencing. The primers and PCR reaction protocol have been described elsewhere (28).

Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normal distribution of each variable. Based on a 3.5-fold difference of adverse outcome in the two groups of patients (TERT promoter mutated versus TERT promoter not mutated) and considering a mean frequency of TERT promoter mutation around 10% in FDTC, as it comes from histological studies, a sample size of at least 341 patients was calculated (α=0.05, β=0.2). The Mann-Whitney test and the Kruskal-Wallis test for

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nonparametric data were used to correlate age at diagnosis and cancer size with final outcome and mutational status, where appropriate. Categorical variables (gender, extrathyroidal extension, multifocality, vascular invasion, lymph node metastases, distant metastases, PTC histological variant, stage at diagnosis) were compared with outcome and mutational status using the chi-square test. Disease-free survival data were analyzed with the Kaplan-Meier method; multivariate analyses of factors affecting metastases and outcome were conducted using logistic regression. A p-value <0.05 was considered statistically significant.

RESULTS

Patients

Of the 436 patients included in our study, 335 (76.8%) were female and 101 (23.2%) were male. The patients were a mean 47.8±13.6 years old (median 47 years). There were 129/436 patients (29.6%) classified as TIR-4, and 307/436 (70.4%) as TIR-5. Among 436 fine-needle aspirates, 423 (97%) were classified as malignant on histopathological review. In detail, the histological classification of the malignant nodules was as follows: 282/423 classical variant of papillary thyroid carcinoma (CV-PTC), 26/423 follicular variant of PTC (FV-PTC), 33/423 oxyphilic variant of PTC (VO-PTC), 70/423 aggressive variants of PTC, 7/423 follicular thyroid carcinoma (FTC), 2/423 poorly-differentiated thyroid carcinoma (PDTC), 2/423 anaplastic thyroid carcinoma (ATC) and 1 medullary thyroid carcinoma (MTC). All benign histologies (11/13 follicular adenomas and 2/13 hyperplastic nodules) were scored as TIR-4 at cytological examination. The size of the malignant nodules ranged from 5 to 60 mm (median 14 mm), and 100/436 (22.9%) were microcarcinomas (largest diameter $\leq$10 mm). Prior to surgery 14/85 (16.5%) of microcarcinomas were N1b. According to the 7th edition of the TNM, 313/423 patients (73.9%) with malignant disease were classified in stage I, 44/423 (10.5%) in stage II, 47/423 (11.1%) in stage III, and 19/423 (4.5%) in

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stage IV at diagnosis. All patients were re-classified according to the 8th edition of the TNM as follows: 356/423 (84.2%) in stage I, 60/423 (14.2%) in stage II, 1/423 (0.2%) in stage III, and 6/423 (1.4%) in stage IV. Eleven (2.6%) of the 423 patients with a malignant histology had metastatic disease. Considering only the cases of FDTC, our series was classified according to the American Thyroid Association (ATA) guidelines of 2009 for the initial stratification of patients' cancer recurrence risk, as modified in 2015 (3): 135/422 (32%) were low risk; 264/422 (65.6%) were intermediate risk; and 23/422 (5.5%) were high risk.

At the end of the follow-up, 318/384 patients (82.8%) had an excellent outcome, 37/384 (9.6%) had an indeterminate response, and 29/384 (7.6%) had biochemically or structurally persistent disease, or had died of their thyroid cancer (in 4/384 cases, 1% of patients, 2 of them with ATC and 2 with PTC), while another 2 patients had died of other causes. For the purposes of our study, patient outcome was classified as: (i) excellent 318/384 (82.8%); or (ii) indeterminate response and biochemically persistent disease (48/384, 12.5%) (iii) structurally persistent disease and death due to thyroid cancer (18/384, 4.7%).

Primary tumor size (p=0.001), extrathyroidal extension (p=0.0007), vascular invasion (p=0.0029), lymph node involvement (p=0.0002) - with N1b carrying a higher risk than N1a (p<0.0001), distant metastases (p<0.0001), advanced stage at diagnosis (according to both the 7th and the 8th editions of the TNM, p<0.0001), and *TERT* promoter mutation (p<0.0001) all correlated significantly with the risk of persistent/recurrent disease or disease-related death (Table 1). At multivariate analysis, only cancer size (OR 1.0459, 95% CI 1.0006 to 1.0932), N1b lymph node metastases (OR 11.7323, 95% CI 2.8167 to 48.8681), and distant metastases (OR 10.5559, 95% CI 1.5767 to 70.6692) predicted persistent disease (i.e. a structurally incomplete response and disease-related death).

BRAF mutation testing

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BRAF mutations were detected in 257/434 thyroid nodules (59.2%). The classic c.1799T>A (p.V600E) mutation was found in 256 cases, and a c.1801A>G mutation (p.K601E) in one. Fifty-four (21%) of the 257 patients carrying a *BRAF* mutation had a TIR-4 cytology, and 203 /257 (79%) had a malignant cytology; they all proved malignant on final pathology review. The results of the univariate analysis are summarized in Table 2. *BRAF* mutation correlated with minimal extrathyroidal extension (p=0.0174), multifocality (p=0.0392), the PTC histological variant (the mutation being more prevalent in the classical and aggressive variants, p<0.0001), and cancer size (the mutation being more frequent in small tumors with a median size of 13 mm in BRAF-mutated tumors as opposed to 15 mm in *BRAF* wild-type tumors, p=0.03). Sixty-three (63.3%) of the 99 microcarcinomas carried a *BRAF* mutation. *BRAF* mutations correlated inversely with distant metastases: they were found in 3/11 patients (27.2%) with distant metastases, and in 254/410 (61.9%) of those without them (p=0.021). *BRAF* mutation status also correlated with a lower incidence of second treatments (p=0.0425), while it was not associated with disease outcome at the end of the follow-up.

TERT **promoter mutation testing**

TERT promoter mutations were detected in 20/436 (4.6%) of cytologies. Nineteen of these 20 patients carried a C228T somatic TERT promoter mutation, and one had a C250T mutation. There was no overlap between the C228T and C250T mutations, the former being more prevalent than the latter. Eleven (55%) of the 20 patients also carried a BRAF mutation. Seven (35%) of the 20 patients carrying a *TERT* promoter mutation had a TIR-4 cytology, while 13 (65%) had a malignant cytology. The histological and clinical characteristics of the patients with *TERT* promoter mutations are given in Table 3. *TERT* promoter mutations were detected in 17/411 of the patients with PTC, in 1/7 of those with FTC, and in (1/2), of those with PDTC and ATC (1/2). The results of the univariate analysis are summarized in Table 2. Following ATA Guidelines, patients with TERT

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promoter mutations were assigned to the following ATA risk categories: 4/20 (20%) were ATA low risk; 12/20 (60%) were intermediate risk; and 4/20 (20%) were high risk. *TERT* promoter mutations correlated with older age at diagnosis (median 68.5 years vs 46 years, p<0.0001), larger-sized primary tumors (25 mm vs 14 mm, p=0.0002, with microcarcinomas all negative for *TERT* promoter mutations), the PTC histological variant (the mutation being more prevalent in the oxyntic and aggressive variants, p=0.0079), advanced stage at diagnosis (p<0.0001), and distant metastases (p<0.0001). Extrathyroidal extension (p=0.542), multifocality (p=0.523), vascular invasion (p=0.315), and lymph node metastases (0.954) did not correlate with *TERT* promoter mutation. When outcome was analyzed, the thyroid disease was structurally persistent, or was the cause of death in 5/19 (26.3%) *TERT* promoter-mutated cancers as opposed to 13 (3.6%) of the 365 *TERT* promoter wild-type tumors (p<0.0001). *TERT* promoter status was not an independent risk factor for disease persistence (structurally incomplete response and disease-related death) at multivariate analysis, however. Interestingly, the frequency of *TERT* promoter mutation increased with worsening outcomes: mutations were present in 11/318 patients (3.5%) with an excellent response, in 3/48 (6.3%)with a biochemically persistent disease or indeterminate response, in 3/14 (21.4%) with structurally incomplete response, and in 4/11 patients (36.4%) with distant metastases, and 2/4 (50%) patients who died. In addition, 6 (31.6%) of 19 patients with *TERT* promoter mutations required further treatment during their follow-up as opposed to 40/386 (10.4%) of the wild-type cases (p=0.045).

Distant metastases

When distant metastases were considered, older age at diagnosis (p=0.0487), larger tumor size (p=0.0015), extrathyroidal extension (p=0.0120), lymph node involvement (both N0 versus N1, and N0+N1a versus N1b; p=0.0117 and p=0.0001, respectively), and *TERT* promoter mutation (p<0.0001) all correlated with M1 status (Table 4). On the other hand, *BRAF*-mutated tumors

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developed distant metastases less frequently than wild-type tumors (p=0.0201). Intriguingly, only *TERT* promoter mutation (OR 40.58; 95% CI 3.06 to 539.04), and N1b (versus N0 and N1a) (OR 40.16, 95% CI 3.48 to 463.04) correlated independently with distant metastases at multivariate analysis. To be more precise as regards lymph node involvement, simply dichotomizing this variable as present or absent (N0 versus N1a and N1b) was unable to independently predict the presence of distant metastases at multivariate analysis.

TERT promoter and BRAF testing

The frequency of combined *BRAF* and *TERT* promoter mutations was also analyzed: 170/436 patients (38.9%) carried no mutations; 246/436 (56.4%) had *BRAF* mutations; 9/436 (2.1%) had *TERT* promoter mutations; and 11/436 (2.5%) had both. The results are summarized in Table 2. The most interesting finding that emerged from this analysis regards the advanced age at diagnosis of patients carrying both mutations, who were significantly older (median 69 years) than patients with single mutations (46.5 and 60 years, respectively, for *BRAF* and *TERT* promoter mutations), or no mutations (45.5 years); $p < 0.0001$.

DISCUSSION

This is the first study, to our knowledge, on the frequency of *TERT* promoter mutations in a large series of suspect or frankly malignant thyroid cytologies. The first interesting finding that emerged concerns the overall frequency of *TERT* promoter mutations in this setting, which was only around 5%. The overall rate of TERT mutations on histologically differentiated thyroid cancer specimens reportedly ranges from 7% to 23% (18-21), and the particularly low rate documented in our series may have several explanations. One important factor to consider is the subclonality of such mutations, which makes them more challenging to ascertain on cytological than on histological

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specimens because genotyping on aspirates may not be representative of whole lesions. Another possible reason lies in the characteristics of our cytological series (obtained prior to surgery), which included a considerable proportion of microcarcinomas (22.9%, all negative for *TERT* promoter mutation), and low-risk cancers (32%), with a consequently low proportion of metastatic diseases. Taking all these concerns into account makes investigating the prognostic value of *TERT* promoter mutation even more intriguing. *TERT* promoter mutations were also found to correlate with older age, larger tumors, distant metastases, advanced tumor stage, persistent disease during the follow-up, and a higher frequency of second treatments (Table 2). The close relationship between *TERT* promoter mutations and the presence of distant metastases is particularly noteworthy from a clinical point of view, as this molecular marker emerged as an independent predictor of distant metastases at multivariate analysis. The association between *TERT* promoter mutation in surgical specimens and metastatic thyroid disease has already been extensively described (6), but our study is the first to demonstrate that this applies when *TERT* promoter mutation is analyzed before any surgery as well. In such a presurgical setting, *TERT* promoter mutation correlated with higher rates of second treatments, and with persistent disease at the end of the follow-up, confirming the data obtained after surgery in the literature (19). Disease persistence also correlated with extrathyroidal extension, vascular invasion, lymph node metastases, distant metastases, and advanced stage, but none of these variables (except for *TERT* promoter mutations and N1b) can be known prior to surgery. Hence the considerable potential of *TERT* promoter analysis as a prognostic molecular marker. The correlation with outcome was lost at multivariate analysis, however, when only cancer size, laterocervical lymph node involvement and distant metastases, but not *TERT* promoter mutation, were independent predictors of persistent disease. It is also worth noting that the rate of *TERT* promoter mutations rose with tumor aggressiveness (and dropped in patients with a good prognosis). Together with the association with older age and larger tumor size, this would suggest that *TERT* promoter mutation is a later genetic event in tumor carcinogenesis, giving tumors a more aggressive potential.

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In short, the most important added value emerging from our study is that, like N1b status, *TERT* promoter mutations found at cytology on malignant and suspect thyroid nodules independently predict distant metastases. The presence of lateral neck compartment lymph node metastases is generally known before surgery, and orients the choice of initial surgical approach: according to recent recommendations (recommendation 35 in the 2015 ATA Guidelines (3)), a more conservative surgical strategy is feasible in patients with clinical N0 disease. TERT promoter mutation testing could be particularly useful in patients without any clinically-evident lymph node involvement and with primary tumors less than 4 cm in size: given its association with distant metastases, a mutated TERT would promptly make such patients candidates for total thyroidectomy followed by ^{131}I administration.

In these times of personalized surgical management and treatment, knowing of a factor that predicts metastases already at the time of a thyroid malignancy's initial diagnosis would be particularly useful for identifying the small subset of aggressive thyroid cancers that warrant more extensive surgery, higher doses of ^{131}I, and a closer follow-up.

A limitation of the present study concerns its retrospective nature, and the low frequency of TERT promoter mutations (5%) in our consecutive series could limit its usefulness in clinical practice. Further data, also from high-risk and preferably prospective series, may help to clarify the cost-effectiveness of TERT mutation testing as a presurgical marker of distant metastases. On the other hand, the subclonality of TERT promoter mutations could result in a low frequency of this finding even in high-risk series. TERT promoter mutations were found more frequently in aggressive and oxyntic (or oncocytic) variants of PTC than in other variants, and we can offer no definitive explanation for this. The oncocytic features of thyroid cells stem from the accumulation of altered mitochondria in the cytoplasm, but the pathogenesis of oncocytic thyroid tumors has yet to be fully elucidated (29). There is plenty of evidence to suggest that oncocytic tumors follow a different genetic pathway from their non-oncocytic counterparts (30, 31, 32). The clinical behavior of this subtype is still controversial, however: it resembled that of typical PTC in many series (29),

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while some authors reported a more aggressive behavior, with higher rates of cancer recurrence and mortality (33, 34).

BRAF mutations *per se* do not seem to be equally useful as a prognostic factor before surgery. In our consecutive series, they did not correlate with persistent disease, while they correlated inversely with distant metastases (Table 4) and the need for second treatments. This picture is in line with the results of other studies (15, 16, 22). It may be that *BRAF* mutations are early events in thyroid carcinogenesis. In fact, they were found in 63.3% of the microcarcinomas in our series (roughly the same proportion as the overall frequency of *BRAF* mutations), and they did not give cancer cells a greater metastatic potential. *BRAF* mutations could only predict the presence of minimal extrathyroidal extension and multifocality. In 55% of the nodules with *TERT* promoter mutations there were *BRAF* mutations too, confirming the association reported in the literature. As for the features of cancers involving both mutations, the combination of a *BRAF* mutation with a *TERT* promoter mutation did not make the cancer more aggressive than a *TERT* promoter mutation alone. Here again, our findings are in line with those coming from other series (19), and from a recent meta-analysis (6). These results should be considered with caution, however, given the small number of patients carrying both mutations. It is worth noting the association between older age and the presence of both mutations. Previous studies have also shown that the cancer-related mutational burden in solid tissue (be it malignant or benign) (35, 36) increases with age, and is one of the hallmarks of senescence.

In conclusion, *TERT* promoter mutations identified on FNA cytology prior to surgery were found to correlate with aggressive phenotypes, although this mutation was not an independent predictor of disease outcome. Even in the cytological setting, *TERT* promoter mutation analysis was able to identify 36.4% of the patients with distant metastases and was thus an independent predictor of M1 status. It could therefore be used as a marker for risk stratification purposes, and to guide a patient's surgical and radioiodine treatment. A possible weakness of such an approach lies in the

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relatively low frequency of *TERT* promoter mutations, which was only around 5% in our series. Obtaining further data from higher-risk series may help to clarify its cost-effectiveness as a pre-surgical marker of distant metastases.

Declaration of interests: Simona Censi, Susi Barollo, Elisabetta Grespan, Sara Watutantrige-Fernando, Jacopo Manso, Maurizio Iacobone, Eric Casal Ide, Francesca Galuppini, Ambrogio Fassina, Gianmaria Pennelli, Loris Bertazza, Federica Vianello and Caterina Mian declare that none have any conflicts of interest that might be perceived as prejudicing the impartiality of the reported research**.**

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	Total	Structurally persistent disease and death from thyroid cancer	Indeterminate response and biochemically persistent disease	Excellent response	*P*-Value
n /total *n* (%)		18/384 (4.7%)	48/384 (12.5%)	318/384 (82.8%)	
Gender					0.889
M	90/384 (23.4%)	4/18(22.2%)	10/48 (20.8%)	76/318 (23.9%)	
F	294/384 (76.6%)	14/18 (77.8%)	38/48 (79.2%)	242/318 (76.1%)	
Median years	47	49	46	47	0.483
Median tumor size mm	14	30	15	14	0.001
Extrathyroidal extension					0.0007
Yes	207/358 (57.8%)	13/14 (92.9%)	33/44 (75%)	161/300 (53.7%)	
No	151/358 (42.2%)	1/14 (7.1%)	11/44 (25%)	139/300 (46.3%)	
Multifocality					0.218
Yes	212/383 (55.4%)	12/17 (70.6%)	30/48 (62.5%)	170/318 (53.5%)	
No	171/383 (44.6%)	5/17 (29.4%)	18/48 (37.5%)	148/318 (46.5%)	
Vascular invasion					0.0029
Yes	130/203 (64%)	11/11 (100%)	22/27 (81.5%)	97/165 (58.8%)	
No	73/203 (36%)	0/11 (0%)	5/27 (18.5%)	68/165 (41.2%)	
PTC histological variants					0.8073
CV	254/373 (68.1%)	8/15 (53.3%)	32/46 (69.6%)	214/312 (68.6%)	
FV	23/373 (6.2%)	1/15 (6.7%)	2/46 (4.3%)	20/312 (6.4%)	
OV	30/373 (8.0%)	1/15 (6.7%)	4/46 (8.7%)	25/312 (8.0%)	
Other, aggressive	66/373 (17.7%)	5/15 (33.3%)	8/46 (17.4%)	53/312 (17%)	
Lymph node metastases					0.0002
Yes	166/334 (49.7%)	14/16 (87.5%)	28/41 (68.3%)	124/277 (44.8%)	
No	168/334 (50.3%)	2/16 (12.5%)	13/41 (31.7%)	153/277 (55.2%)	
Lymph node metastases					<0.0001
N0	168/334 (50.3%)	2/16 (12.5%)	13/41 (31.7%)	153/277 (55.2%)	
N1a	100/334 (29.9%)	2/16 (12.5%)	15/41 (36.6%)	83/277 (30%)	
N1b	66/334 (19.8%)	12/16 (75.0%)	13/41 (31.7%)	41/277 (14.8%)	
Distant metastases					<0.0001
Yes	11/384 (2.9%)	7/18 (38.9%)	3/48 (6.2%)	1/318 (0.3%)	
No	373/384 (97.1%)	11/18 (61.1%)	45/48 (93.7%)	317/318 (99.7%)	
TNM stage 7th edition					<0.0001

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	I	283/384 (73.7%)	6/18 (33.3%)	36/48 (75%)	241/318 (75.8%)	
	II	39/384 (10.2%)	7/18 (38.9%)	6/48 (12.5%)	26/318 (8.2%)	
	III	45/384 (11.7%)	0/18 (0%)	3/48 (6.2%)	42/318 (13.2%)	
	IV	17/384 (4.4%)	5/18 (27.8%)	3/48 (6.2%)	9/318 (2.8%)	
TNM stage 8th edition						<0.0001
	I	325/384 (84.6%)	8/18 (44.4%)	37/48 (77.1%)	280/318 (88.1%)	
	II	53/384 (13.8%)	7/18 (38.9%)	10/48 (20.8%)	36/318 (11.3%)	
	III	1/384 (0.3%)	0/18 (0%)	0/48 (0%)	1/318 (0.3%)	
	IV	5/384 (1.3%)	3/18 (16.7%)	1/48 (2.1%)	1/318 (0.3%)	
TERT promoter mutation						<0.0001
	Yes	19/384	5/18 (27.8%)	3/48 (93.7%)	11/318 (3.5%)	
	No	365/384	13/18 (72.2%)	45/48 (6.2%)	307/318 (96.5%)	
BRAF mutation						0.5347
	Yes	237/382 (62%)	9/18 (50%)	31/48 (64.6%)	197/316 (62.3%)	
	No	145/382 (38%)	9/18 (50%)	17/48 (35.4%)	119/316 (37.7%)	

Table 1: Correlation between structural incomplete response and thyroid cancer related death versus biochemical incomplete response and indeterminate response versus excellent response and clinicopathological features of PTC and molecular status (univariate analysis).

Notes: CV-PTC: classical variant of papillary thyroid carcinoma; FV-PTC: follicular variant of papillary thyroid carcinoma; VO-PTC: oxyphilic variant of papillary thyroid carcinoma.

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		Total	BRAF mutated	BRAF wild type	*P*-Value	TERT promoter mutated	TERT promoter wild-type	P-Value	BRAF and TERT promoter mutated	BRAF and TERT promoter wild-type	*P*-Value
Gender					0.778			0.732			0.966
	M	100/434 (23%)	58/100 (58%)	42/100 (42%)		4/101 (4%)	97/101 (96%)		2/101 (2%)	41/101 (40.6%)	
	F	334/434 (77%)	199/334 (59.6%)	135/334 (40.4%)		16/335 (4.8)	319/335 (95.2)		9/225 (2.7%)	129/335 (38.5%)	
Median age, years		47	47	46	0.878	68.5	46	<0.0001	69	45.5	<0.001
Median tumor size, mm		14	13	15	0.03	25	14	0.0002	17	15	<0.0001
Extrathyroidal extension					0.0174			0.542			0.04
	Yes	220/392 (56.1%)	145/220 (65.9%)	72/220 (34.1%)		9/222 (4.1%)	213/222 (95.9%)		7/222 (3.2%)	75/222 (33.8%)	
	No	172/392 (43.9%)	93/172 (54.1%)	79/172 (45.9%)		5/172 (2.9%)	167/172 (97.1%)		1/172 (0.6%)	75/172 (43.6%)	
Multifocality					0.0392			0.523			0.04
	Yes	230/420 (54.8%)	151/230 (65.7%)	79/230 (34.3%)		9/230 (3.9%)	221/230 (96.1%)		4/230 (1.7%)	74/230 (32.2%)	
	No	190/420 (45.2%)	106/190 (55.7%)	84/190 (44.3%)		10/192 (5.2%)	182/192 (94.8%)		7/192 (3.6%)	83/192 (43.2%)	
Vascular invasion					0.8495			0.315			0.47
	Yes	137/223 (61.4%)	83/137 (60.5%)	54/137 (39.5%)		7/139 (5.0%)	132/139 (95%)		4/139 (2.9%)	53/139 (38.1%)	
	No	86/223 (38.6%)	51/86 (59.3%)	35/86 (40.7%)		2/86 (2.3%)	84/86 (97.7%)		0/86 (0%)	33/86 (38.4%)	
Histological variants					<0.0001			0.0079			<0.001
	CV	281/409 (68.7%)	186/281 (66.2%)	95/281 (33.8%)		7/282 (2.5%)	275/282 (97.5%)		5/357 (1.4%)	93/282 (33%)	
	FV	26/409 (6.4%)	5/26 (19.2%)	21/26 (80.8%)		0/26 (0%)	26/26 (100%)		5/59 (8.5%)	21/26 (80.8%)	
	OV	32/409 (7.8%)	18/32 (56.3%)	14/32 (43.7%)		4/33 (12.1%)	29/33 (87.9%)		0/1 (0%)	14/33 (42.2%)	
	AggrV	70/409 (17.1%)	48/70 (68.6%)	22/70 (31.4%)		6/70 (8.6%)	64/70 (91.4%)		1/6 (16.7%)	20/70 (28.6%)	
Lymph node metastases					0.485			0.954			0.859
	Yes	173/363 (47.7%)	104/173 (60.1%)	69/173 (39.9%)		7/173 (4.0%)	166/173 (96%)		3/173 (1.7%)	65/173 (37.6%)	
	No	190/363 (52.3%)	121/190 (63.6%)	69/190 (36.4%)		8/192 (4.2%)	184/192 (95.8%)		5/192 (2.6%)	68/192 (35.4%)	
Lymph node metastases					0.1325			0.6182			0.422
	N0	190/363 (52.3%)	121/190 (63.7%)	69/190 (36.3%)		8/192 (4.2%)	184/192 (95.8%)		5/192 (2.6%)	68/192 (35.4%)	
	N1a	105/363 (28.9%)	69/105 (65.7%)	36/105 (34.3%)		3/105 (2.9%)	102/105 (97.1%)		2/105 (1.9%)	35/105 (33.3%)	
	N1b	68/363 (18.7%)	35/68 (51.5%)	33/68 (48.5%)		4/68 (5.9%)	64/68 (94.1%)		1/68 (1.5%)	30/68 (44.1%)	
Distant metastases					0.0201			<0.0001			<0.0001
	Yes	11/421 (2.6%)	3/11 (27.2%)	8/11 (72.8)		4/11 (36.4%)	7/11 (63.6%)		2/11 (18.2%)	6/11 (54.5%)	
	No	410/421 (97.4%)	254/410 (61.9%)	156/410 (38.1)		16/412 (3.8%)	396/412 (96.2%)		9/412 (2.2%)	151/412 (36.7%)	
TNM stage (8th edition)					0.0533			<0.0001			
	I	355/421 (84.3%)	216/355 (60.8%)	139/355 (39.2%)		9/357 (2.5%)	348/357 (97.5%)		5/357 (1.4%)	137/357 (38.4%)	
	II	59/421 (14%)	40/59 (67.8%)	19/59 (32.2%)		8/59 (13.6%)	51/59 (%)		5/59 (8.5%)	16/59 (27.1%)	
	III	1/421 (0.2%)	0/1 (0%)	1/1 (100%)		1/1 (100%)	0/1 (%)		0/1 (0%)	0/1 (0%)	
	IV	6/421 (1.4%)	1/6 (16.7%)	5/6 (83.3%)		2/6 (33.3%)	4/6 66.7(%)		1/6 (16.7%)	4/6 (66.7%)	
Outcome					0.5347			<0.0001			<0.0001
	Excellent response	318/384 (82.8%)	197/316 (62.3%)	119/316 (37.7%)		11/318 (3.5%)	307/318 (96.5%)		7/318 (2.2%)	117/318 (36.8%)	
	Indeterminate response and biochemically persistent disease	48/384, (12.5%)	31/48 (64.6%)	17/48 (35.4%)		3/48 (93.7%)	45/48 (6.2%)		3/48 (6.2%)	17/48 (35.4%)	
	Structurally persistent disease and death from	18/384, (4.7%)	9/18 (50%)	9/18 (50%)		5/18 (27.8%)	13/18 (72.2%)		1/18 (5.6%)	5/18 (27.8%)	

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	thyroid cancer										
Second treatment					0.0425			0.045			0.0076
	Yes	46/403 (11.4%)	22/46 (47.8%)	24/46 (52.2%)		6/46 (13.0%)	40/46 (87.0%)		3/46 (6.5%)	21/46 (45.7%)	
	No	357/403 (88.6%)	226/357 (63.3%)	130/355 (36.7%)		13/359 (3.6%)	346/359 (96.4%)		8/359 (2.2%)	128/359 (45.7%)	

Table 2: correlation between TERT promoter and BRAF mutations, alone or combined and clinicopathological features and final outcome (univariate analysis).
Notes: CV-PTC: classical variant of papillary thyroid carcinoma; FV-PTC: follicular variant of papillary thyroid carcinoma; VO-PTC: oxyphilic variant of papillary thyroid carcinoma.

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Pz	TERT mutation	Age/Sex	BRAF mutation	Histology	Cancer size (mm)	T	N	M	^{131}I ablation/dose (mCi)	Other treatments	FU (months)	Outcome
1	C228T	75/F	No	PTC-TC	48	3	N1b	M1	Yes/150	Yes/^{131}I	28	Death
2	C228T	60/M	No	PTC-CV	55	4	N1b	M0	Yes/150	No	91	Excellent
3	C228T	76/F	Yes	PTC-CV	60	3	Nx	M0	Yes/100	No	108	Excellent
4	C250T	65/F	No	PTC-OV	22	2	N1a	M0	Yes/150	No	81	Excellent
5	C225T	44/M	No	PTC-CV	28	2	Nx	M0	Yes/100	No	89	Excellent
6	C225T	69/F	Yes	PTC-OV	28	2	N0	M1	Yes/200	Yes/RTE	88	Biochemical incomplete response
7	C228T	69/F	Yes	PTC-TC	13	1	N0	M0	Yes/150	No	87	Excellent
8	C228T	48/F	No	PTC-Ho	24	2	N1b	M0	Yes/150	Yes/131I	54	Structural incomplete
9	C228T	60/F	Yes	PTC-OV	38	2	N1a	M0	Yes/100	No	91	Indeterminate
10	C228T	56/F	Yes	PTV-OV	12	1	N0	M0	Yes/150	No	111	Excellent
11	C228T	80/F	Yes	PTC-CV	19	1	Nx	M0	Yes/100	No	35	Excellent
12	C228T	48/F	No	PTC-CV	nd	1	N0	M0	No	na	na	na
13	C228T	54/F	No	ATC	na	na	na	M1	No	RTE and TKI	19	Death
14	C228T	81/F	Yes	PTC-TC	14	1	No	M0	No	No	19	Excellent
15	C228T	92/F	No	FTC	43	3	No	Mo	No	No	58	Structural incomplete
16	C228T	69/F	Yes	PTC-CV	15	1	Nx	M0	Yes/100	Yes/laterocervical surgery and RTE	167	Biochemical incomplete

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17	*C228T*	*75/M*	*Yes*	*PTC-TC*	*34*	*2*	*N1a*	*M0*	*Yes/100*	*No*	*54*	*Excellent*
18	*C228T*	*72/F*	*Yes*	*PTC-CV*	*15*	*1*	*N0*	*M0*	*No*	*No*	*44*	*Excellent*
19	*C228T*	*50/M*	*Yes*	*PTC-TC*	*17*	*4*	*N1b*	*M1*	*Yes/150*	*RTE and TKI*	*10*	*Structural incomplete*
20	*C228T*	*68/F*	*No*	*PDTC*	*25*	*2*	*N0*	*M0*	*Yes/100*	*No*	*43*	*Excellent*

Table 3: Clinicopathological characteristics of *TERT* promoter-mutated patients. Abbreviations: ATC: anaplastic thyroid carcinoma; F: female; FTC: follicular thyroid carcinoma; M: male; PDTC: poorly differentiated thyroid cancer; pz: patient; PTC-CV: classical variant of papillary thyroid carcinoma; PTC-Ho: hobnail variant of papillary thryoid carcinoma; PTC-OV: oxyntic variant of papillary thyroid carcinoma; PTC-TC: tall cell variant of papillary thyroid carcinoma; RTE: external radio therapy, TKI: tirosin kinase inhibitors.

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		Total	M1	M0	*P*-Value
Gender					0.718
	M	96/423 (22.7%)	2/96 (2.1%)	94/96 (97.9%)	
	F	327/423 (77.3%)	9/327 (2.8%)	318/327 (97.2%)	
Median Age, years		47	54	46	0.0487
Median Tumor size, mm		14	28	14	0.0015
Extrathyroidal extension					0.0120
	Yes	222/394 (56.3%)	8/222 (3.6%)	214/222 (96.4%)	
	No	172/394 (43.7%)	172/172 (100%)	0/172 (0%)	
Multifocality					0.3198
	Yes	230/422 (54.5%)	7/230 (3%)	223/230 (97%)	
	No	192/422 (45.5%)	3/192 (1.6%)	189/192 (98.4%)	
Vascular invasion					0.5838
	Yes	139/225 (61.8%)	3/139 (2.2%)	136/139 (97.8%)	
	No	86/225 (38.2%)	1/86 (1.2%)	85/86 (98.8%)	
PTC histological variants					0.502
	CV	282/411 (6.6%)	5/282 (1.8%)	277/282 (98.2%)	
	FV	26/411 (6.3%)	0/26 (0%)	26/26 (100%)	
	OV	33/411 (8.0%)	1/33 (3%)	32/33 (97%)	
	Other, aggressive	70/411 (17.0%)	3/70 (4.3%)	67/70 (95.7%)	
Lymph node metastases					0.0117
	N0	192/365 (52.6%)	1/192 (0.5%)	191/192 (99.5%)	
	N1a and N1b	173/365 (47.4%)	8/173 (4.6%)	165/173 (95.4%)	
Lymph node metastases					0.0001
	N0	192/365 (52.6%)	1/192 (0.5%)	191/192 (99.5%)	
	N1a	105/365 (28.8%)	1/105 (1%)	104/105 (99%)	
	N1b	68/365 (52.6%)	7/68 (10.3%)	61/68 (89.7%)	
TERT promoter mutation					<0.0001
	Yes	20/423 (4.7%)	4/20 (20%)	16/20 (80%)	
	No	403/423 (95.3%)	7/403 (1.7%)	396/403 (98.3%)	
BRAF mutation					0.0201
	Yes	257/421 (61%)	3/257 (1.2%)	254/257 (98.8%)	
	No	164/421 (39%)	8/164 (4.9%)	156/164 (95.1%)	

Table 4: Correlation between metastatic disease and clinicopathological and molecular features (univariate analysis).
Notes: CV-PTC: classical variant of papillary thyroid carcinoma; FV-PTC: follicular variant of papillary thyroid carcinoma; VO-PTC: oxyphilic variant of papillary thyroid carcinoma.

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