Circulating *BRAF*^{V600E} in the Diagnosis and Follow-Up of Differentiated Papillary Thyroid Carcinoma

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Context: Cell-free nucleic acids circulating in plasma are considered a promising noninvasive tool for cancer monitoring. *BRAF*^{V600E} mutation in cell-free DNA (cfDNA) could represent an appropriate marker for papillary thyroid carcinoma (PTC).

Objective: Our aim is to investigate the role of *BRAF*^{V600E}-mutated allele in cfDNA as a marker for the diagnosis and follow-up of PTC.

Study Design: *BRAF*^{V600E} allele was detected and quantified by an allele-specific real-time quantitative PCR assay in plasma from 103 patients affected by nodular goiter. As control populations, we enrolled 49 healthy subjects and 16 patients with non-nodular thyroid diseases.

Results: The percentage of circulating *BRAF*^{V600E} was significantly different between patients and controls and throughout different cytological categories of ultrasound-assisted fine-needle aspiration. Patients with a histopathological diagnosis of PTC showed a higher percentage of circulating *BRAF*^{V600E} ($P = .035$) compared to those with benign histology. In 19 patients, a second blood draw, taken 3–6 months after surgery, showed a lower percentage of *BRAF*^{V600E} in cfDNA than the presurgical sample ($P < .001$). The diagnostic performance of circulating *BRAF*^{V600E} was assessed by receiver operating characteristic curve analysis resulting in an area under the curve of 0.797. A cutoff value was chosen corresponding to maximum specificity (65%) and sensitivity (80%). On this basis, we evaluated the predictive value of *BRAF*^{V600E} in Thy 3 patients with a resulting positive predictive value of 33% and a negative predictive value of 80%.

Conclusions: The results of the present study provide encouraging data supporting the possibility to take advantage of circulating *BRAF*^{V600E} in the management of PTC. **(*J Clin Endocrinol Metab* 98: 3359–3365, 2013)**

Cancer accounts for 5–15% of all thyroid nodules, and most of these carcinomas consist of differentiated forms originating from follicular cells, mainly papillary thyroid carcinoma (PTC) (1–3). Although 5–10% of locoregional recurrences have been reported, these tumors exhibit low biological aggressiveness and are associated with a high rate of survival (4). As detailed in recent guidelines (5, 6), the treatment of PTC consists of thyroidectomy followed, when appropriate, by radioactive iodine (RAI) treatment with remnant ablation and adjuvant aims. The patients are subsequently followed up by means of ultrasound examination of the neck and measurement

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Abbreviations: cfDNA, cell-free DNA; FFPE, formalin-fixed, paraffin-embedded; FNA, fine-needle aspiration; NNT, non-nodular thyroid disease; PTC, papillary thyroid carcinoma; RAI, radioactive iodine; ROC, receiver operating characteristic; Tg, thyroglobulin; US-FNA, ultrasound-assisted FNA.

of the circulating thyroglobulin (Tg), thyroid-specific protein involved in the synthesis of thyroid hormones, in the absence of anti-Tg-specific antibodies. In the minority of patients where advanced disease is identified RAI, surgery, external radiotherapy, or chemotherapy may be used (7).

Based on these data, clinical efforts must be undertaken to avoid overtreatment of benign nodules and to identify patients with potentially more aggressive disease to be submitted to more stringent therapy and follow-up.

The diagnostic procedures of thyroid nodules include ultrasound examination of the neck and cytological analysis of fine-needle aspiration (FNA) (8). Although ultrasound features may help in identifying thyroid nodules harboring a differentiated carcinoma, FNA has shown the highest sensitivity and specificity (8). However, 20% of nodules are diagnosed as indeterminate by cytological examination of FNA, and among this cytological category, only 20–30% of the patients will have thyroid cancer (8). Numerous recent studies assess the importance of integrating the cytological results with the molecular analysis of tumor markers (9–11). Somatic mutations in protooncogenes, such as BRAF, KRAS, HRAS, NRAS, as well as chromosomal alterations, such as RET/PTC rearrangements and PAX/PPARγ translocation, strongly suggest the presence of malignancy (12), thus allowing us to select patients to be submitted to surgery.

The identification of the molecular profile of tumor cells may also have a prognostic value because numerous studies seem to suggest that thyroid carcinomas presenting $BRAF^{V600E}$ mutation are more aggressive and metastasize more frequently (13, 14).

In addition to cytological markers, tumor-specific markers circulating in plasma may represent a noninvasive approach to provide important information in the setting of both diagnosis and follow-up of PTC (15–17).

In fact, the study of circulating nucleic acids is a relatively new but rapidly expanding field of medical research (18). With the increasing demand for noninvasive approaches in the monitoring of cancer, circulating nucleic acids have been considered a promising tool for many clinical applications that can be achieved with the advances in technology and detection methods and improvements in sensitivity and specificity of the detection.

BRAF somatic mutations have been reported in a wide range of human cancers, with the highest frequency in melanoma and thyroid cancer (19).

Because $BRAF^{V600E}$ is one of the most represented somatic mutations in PTC (20), it would be an appropriate circulating marker for this disease.

Numerous studies have been published identifying $BRAF^{V600E}$ somatic mutation in cytological samples of thyroid cancer, whereas few data are available on the possibility of reliably detecting BRAF-mutated DNA in blood (21–23). Starting from the first results reported in different types of cancer (21), BRAF positivity in blood seems to significantly correlate to the presence of active disease (22, 23).

The use of $BRAF^{V600E}$ as a circulating marker could have several important clinical applications. In the diagnostic setting, it would integrate the data obtained from cytological analysis of FNA, providing more complete information in particular cases such as multinodular goiters where it is not possible to sample all nodules by FNA.

The availability of circulating markers could also play an important role in the short- and long-term follow-up of patients with previous thyroid cancer. Tg is an extremely sensitive and reliable marker currently used for monitoring persistent or recurrent disease in thyroid cancer (24). However, in 25–30% of patients, the use of this marker is hampered by the presence of Tg antibodies (25). Moreover, in patients that did not undergo a complete thyroid ablation by surgical and RAI ablation, Tg is less powerful in predicting persistency or recurrence of thyroid cancer (5, 6). In these cases, alternative circulating markers would have a great impact in the assessment of residual disease.

We therefore undertake the present study to explore the possibility of using $BRAF^{V600E}$ mutation as a marker associated to cell-free DNA (cfDNA) for the diagnosis and follow-up of PTC.

Patients and Methods

Patients

A total of 103 patients (74 females and 29 males; age range, 18–90 y; median, 55 y) admitted to the Endocrinology Unit of Careggi Teaching Hospital because of nodular goiter were evaluated for the presence of $BRAF^{V600E}$-mutated allele in cfDNA circulating in plasma.

Patients were submitted to ultrasound-assisted FNA (US-FNA) according to recent guidelines (8), and the cytological diagnoses were made in accordance with the 5 diagnostic groups of the British Thyroid Association 2007, namely: Thy 1, nondiagnostic; Thy 2, non-neoplastic; Thy 3, follicular lesions; Thy 4, suspicious of malignancy; and Thy 5, diagnostic of malignancy (26). When more than 1 nodule was sampled by US-FNA in multinodular goiters, the worst cytology was considered.

Our case study is composed as follows: Thy 2, n = 27; Thy 3, n = 47; Thy 4, n = 25; and Thy 5, n = 4.

Surgical therapy was suggested to all the patients with Thy 3, Thy 4, and Thy 5, and for 69 of them the histology report was available. Histological examination demonstrated the presence of differentiated thyroid cancer in 38 of 69 patients (all cancers were papillary, 25 were classical variant, 11 were follicular variant, and 2 were minor variants). The 7 patients whose histology was not available belong to the Thy 3 cytology group. Four of them refused thyroidectomy and dropped out of the follow-up. The remaining 3 patients did not undergo surgery because of age

or comorbidities, and they are currently performing ultrasound follow-up without evidence of evolution of the ultrasound pattern.

In 22 subjects, paired formalin-fixed, paraffin-embedded (FFPE) tissues were available and were analyzed for $BRAF^{V600E}$. Only FFPE tumor tissues containing at least 70% of tumor cells were included.

Nineteen patients affected by PTC were submitted to a second blood draw 3–6 months after surgery (mean, 4.4 mo; range, 2.7–6.4 mo). In this period, 12 patients also underwent RAI treatment according to recent guidelines (5, 6). There was no correlation between the percentage of circulating $BRAF^{V600E}$ and the time from surgery or RAI treatment (data not shown).

As control populations, healthy subjects (n = 49; 26 females and 23 males; age range, 25–89 y; median, 53 y) and patients with non-nodular thyroid diseases (NNT; n = 16; 10 females and 6 males; age range, 23–77 y; median, 53 y) were enrolled in the study. The NNT group includes patients affected by Graves' disease (n = 7), autoimmune hypothyroidism (n = 5), and postoperative hypothyroidism (n = 4).

The research protocol was conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the review board of the Department of Biomedical, Experimental, and Clinical Sciences of the University of Florence; all of the patients signed an informed consent.

DNA extraction

Peripheral blood (5 ml) was collected in an EDTA tube, transported within 1 hour to the laboratory, and centrifuged twice at 4°C for 10 minutes (1.600 and 14.000 relative centrifugal force). Plasma aliquots were stored at −80°C before use. DNA was extracted from 500 μl plasma, using the QIAamp DSP Virus Kit (QIAGEN, Hilden, Germany) and RNAse digestion to prevent RNA interference during assay reaction. DNA was extracted from cell lines by the QIAamp DNA Blood Mini kit (QIAGEN). DNA from FFPE tissues was extracted using the FFPE Tissue kit (QIAGEN).

Detection of $BRAF^{V600E}$ mutation in cfDNA and FFPE tissue samples

For the detection of $BRAF^{V600E}$-mutated allele in cfDNA, we used an allele-specific real-time quantitative PCR assay as previously described (27). Results are reported as percentage of $BRAF^{V600E}$-mutated allele over the wild-type allele.

Statistical analysis

Statistical analysis was carried out using the SPSS software package 19.0 (SPSS, Chicago, Illinois). Statistical differences between qualitative results were assessed by the Fisher's exact test, whereas quantitative data were evaluated by the Student's *t* test for paired or unpaired data.

P values lower than .05 were considered statistically significant. Receiver operating characteristic (ROC) curve analysis based on Thy 4 plus Thy 5 cytologies (all positive for carcinoma at histology) and healthy subjects was exploited for the assessment of the accuracy and identification of a threshold. The Youden Index (defined as Y = sensitivity + specificity − 1) was used for the selection of the optimal threshold value (cutoff point) for the marker under study (28, 29).

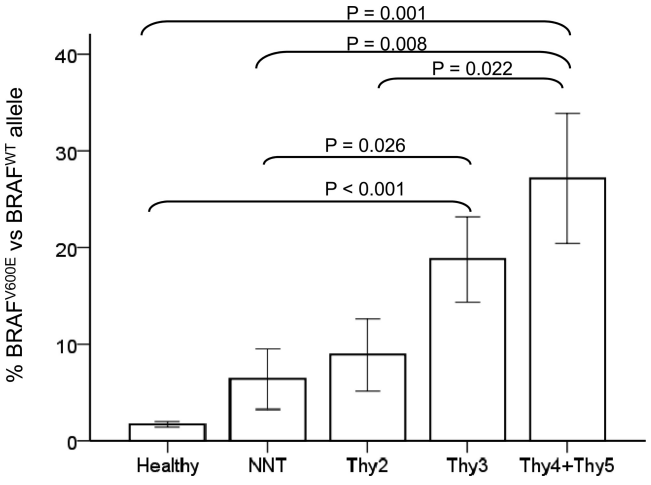


Figure 1. Percentage of $BRAF^{V600E}$ allele vs *BRAF* wild-type (WT) allele (mean ± SE) in cfDNA from patients belonging to different US-FNA cytological categories (Thy 2, n = 27; Thy 3, n = 47; Thy 4 + Thy 5, n = 29), healthy subjects (n = 49), and patients with NNT (n = 16).

Results

Quantitative evaluation of the results

$BRAF^{V600E}$ percentage in plasma: correlation with cytology

Patients affected by nodular thyroid disease were sorted on the basis of the cytological analysis, and the percentage of $BRAF^{V600E}$ in cfDNA was compared to that of healthy subjects and patients with NNT (Figure 1).

The percentage of $BRAF^{V600E}$ in cfDNA was 1.7 ± 0.3 in healthy subjects and 6.4 ± 3.1 in patients with NNT (Figure 1).

Compared to healthy subjects, the percentage of circulating $BRAF^{V600E}$ was significantly higher in patients with Thy 3 (18.7 ± 4.4; $P < .001$) and with Thy 4 plus Thy 5 nodules (27.1 ± 6.7; $P = .001$).

Control subjects affected by NNTs had a lower percentage of $BRAF^{V600E}$ in plasma than Thy 3 patients ($P = .026$) and Thy 4/Thy 5 patients ($P = .008$).

Moreover, subjects with Thy 2 showed a lower percentage of circulating $BRAF^{V600E}$ alleles (8.9 ± 3.7) than Thy 4/Thy 5 patients ($P = .022$).

$BRAF^{V600E}$ percentage before and after surgery in patients affected by thyroid cancer

A group of 19 patients diagnosed with PTC were submitted to an additional blood draw 3–6 months after surgery and after RAI treatment when appropriate. Circulating $BRAF^{V600E}$ percentage was significantly reduced in the postsurgical blood draw (6.5 ± 3.7 vs 43.2 ± 8.9; $P < .001$) compared to presurgical samples (Figure 2).

$BRAF^{V600E}$ percentage in plasma: correlation with histology

$BRAF^{V600E}$ percentage in cfDNA from patients with benign lesions according to histology report (9.9 ± 3.2%;

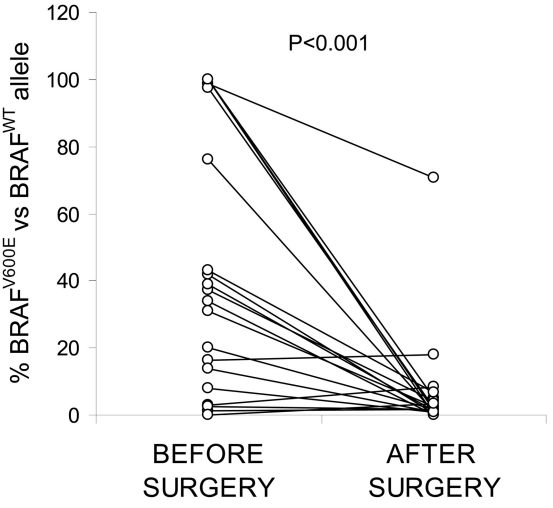


Figure 2. Percentage of *BRAF*^{V600E} allele vs *BRAF* wild-type (WT) allele in cfDNA before and 3–6 months after surgery in 19 patients with histological diagnosis of PTC. Twelve patients were also submitted to RAI.

n = 31) was significantly lower with respect to those with histological evidence of PTC (23.7 ± 5.4%; n = 38; P = .035) (Figure 3).

BRAF^{V600E} percentage in cfDNA is higher in patients affected by classical papillary carcinoma (n = 25) than in those with the follicular variant (n = 11) (31.8 ± 7.3 vs 9.4 ± 6.8; P = .033).

Clinical penetrance of the measurement of plasma *BRAF*^{V600E}

ROC curve analysis was used to assess the performance of circulating *BRAF*^{V600E} determination in thyroid carcinoma patients and healthy controls.

The area under the ROC curve for Thy 4/Thy 5 patients (all positive at histological evaluation) vs healthy subjects was 0.797 (Figure 4). According to this analysis, we could define a cutoff value for *BRAF*^{V600E}-mutated allele in cfDNA to maximize the clinical sensitivity and specificity of the test. The calculated cutoff value was 2.65%, corresponding to 65% sensitivity and 80% specificity.

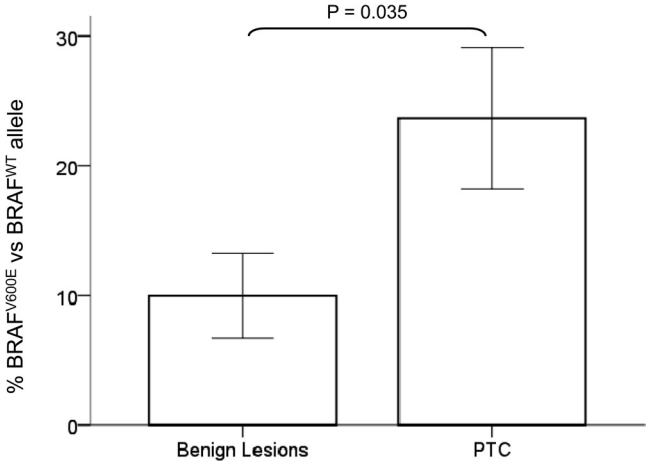
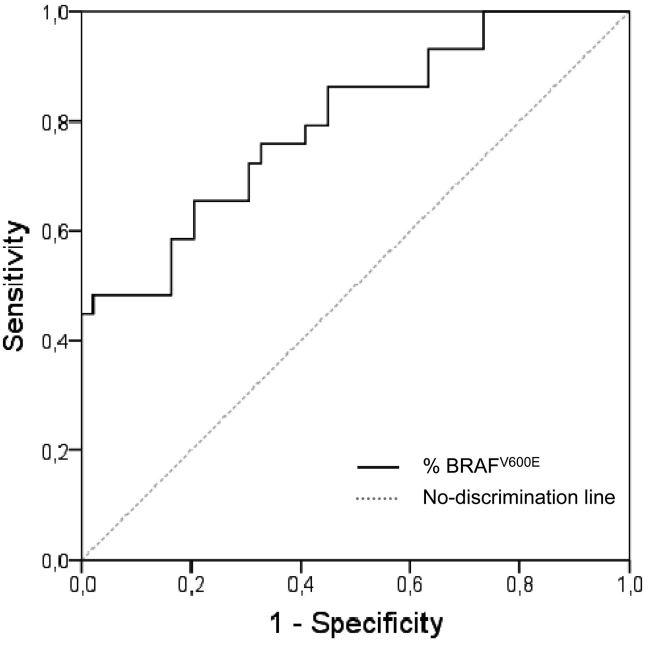


Figure 3. Percentage of *BRAF*^{V600E} allele vs *BRAF* wild-type (WT) allele (mean ± SE) in patients with histological diagnosis of PTC (n = 38) or benign nodules (n = 31).



Area	SE[a]	P value	95% Confidence interval	
			Lower limit	Upper limit
0.797	0.053	<0.001	0.693	0.900

Figure 4. ROC curve derived from the univariate logistic analysis corresponding to *BRAF*^{V600E} percentage in cfDNA in Thy 4 plus Thy 5 cytologies (all positive for PTC at histology, n = 29) and healthy subjects (n = 49). ROC curve parameters, are reported in the table at the bottom of figure.

Qualitative evaluation of the results

All the analyses previously performed using quantitative data are repeated here from a qualitative point of view by means of the determined cutoff.

Qualitative evaluation of *BRAF*^{V600E} in Thy 3 patients

The predictive value of *BRAF*^{V600E} was evaluated in the Thy 3 group, because Thy 4 and Thy 5 cytology is highly informative of the presence of thyroid cancer.

According to the chosen cutoff, 18 of 33 Thy 3 patients were positive for *BRAF*^{V600E} variant. Fisher's exact test revealed that 6 of 9 (67%) histology-positive samples were mutated in *BRAF*, whereas the same variant was found in only 50% (12 of 24) of histology-negative patients (P = .458).

The resulting positive predictive value and negative predictive value for this group of patients were 33 and 80%, respectively.

Qualitative evaluation of *BRAF*^{V600E} before and after surgery in patients affected by thyroid cancer

The qualitative evaluation of the results obtained in 19 patients who had a matched pair of blood samples collected before and after surgery is illustrated in Figure 2. Twelve of 17 (71%) patients were preoperatively positive for mutated *BRAF*^{V600E} and turned negative after surgery. Five patients that were positive before surgery confirmed the same result in the second blood sample collected after treatment. Among these patients, 3 showed a weak positivity and did not have clinical, serological, or instrumental evidence of disease, whereas in 2 patients that remained markedly positive, persistence of disease was highly probable (incomplete resection of the tumor in 1 case and stable detectable serum Tg in the other case).

The 2 patients who were negative for mutated *BRAF*^{V600E} before surgery remained negative after treatment.

Qualitative evaluation of *BRAF*^{V600E} percentage in plasma: correlation with histology

When considering the different histological types of the tumors, we found that the percentage of positive cases was higher in patients affected by classical papillary carcinoma (18 of 25, or 72%) than in subjects bearing the follicular variant (6 of 11, or 54%; $P = .446$)

BRAF^{V600E} in paired FFPE tissues and plasma samples

Comparison of plasma and tissue results in the same subject was obtained in 22 patients by evaluating results of the percentage of *BRAF*^{V600E}-mutated allele on the basis of the above-reported cutoff value. A statistically significant correlation was found when analyzing the concordance between plasma and tissue samples by Fisher's exact test ($P = .006$) (Table 1). Overall, 17 subjects obtained concordant results on tissue as well as plasma, 6 resulting wild-type and 11 showing the mutation in both samples. Two mutated tissues did not find correspondence in plasma DNA, whereas 3 subjects showed the *BRAF*^{V600E} mutation only in plasma.

Table 1. Comparison of *BRAF* ^{V600E} Detection in Plasma and Corresponding FFPE Tissue Samples ($P = .006$, Fisher's exact test).

FFPE Tissue	Plasma		
	Negative	Positive	Total
Negative	7	3	10
Positive	1	11	12
Total	8	14	22

Postsurgery Tg levels in PTC patients with *BRAF*^{V600E} evaluation both in plasma and tissue were always undetectable; thus, this marker was not able to provide additional information in the cases of discordant *BRAF* status.

Discussion

The results of the present study suggest that circulating *BRAF*^{V600E} may be a helpful tool in the diagnosis and follow-up of PTC.

US-FNA is extremely reliable in identifying the small percentage (5–15%) of thyroid cancer among most benign nodular lesions (8). However, in approximately 20% of all US-FNA, cytological examination will not be informative (Thy 3), and the patients will be submitted to surgery. In this cytological category, only 20% of patients will be proven to harbor a cancer that justifies surgical treatment.

The identification of somatic mutations in thyroid carcinoma and the possibility to detect their presence in cytological samples improves the accuracy of US-FNA in the Thy 3 group of patients (10, 11, 30). Among these biomolecular markers, *BRAF*^{V600E} is the most common somatic mutation in PTC, accounting for 45–80% of all genetic variants (19).

During the last 10 years, researchers have been studying cfDNA present in plasma with great expectations on their use as potential biomarkers for cancer and other pathological conditions. cfDNA may derive from apoptotic or necrotic cells and can be released actively from normal and diseased cells. Tumor-specific alterations, such as mutations, can be found in cfDNA of tumoral origin. Methods with high analytical sensitivity are essential for detecting these specific alterations in a background of "normal" cfDNA molecules (31).

Because the presence of circulating *BRAF*^{V600E} was reported in the blood of patients with different types of cancers including thyroid carcinoma (21), few evidences were provided supporting its use in the diagnosis and follow-up of this disease (22, 23). The presence of mutated *BRAF*^{V600E} DNA was reported in 36% of a small series of 14 patients diagnosed with PTC (23). Data obtained from patients submitted to follow-up for PTC suggested a correlation between the detection of circulating cell-free *BRAF*^{V600E} and the presence of active disease (22). At variance with our results and with previous reports, recent data failed to detect circulating *BRAF*^{V600E} in 94 serum samples from patients with PTC positive for the *BRAF*^{V600E} mutation in the tumor itself by using a quantitative PCR method (32). The use of assay reagents with inadequate sensitivity and/or not optimized for plasma samples in addition to uncontrolled preanalytical steps

can be the source of the discordant results on the measurement of $BRAF^{V600E}$-mutated allele in cfDNA. We demonstrated that the percentage of $BRAF^{V600E}$ in cfDNA increased progressively in a significant manner across cytological categories, and it was higher in patients with histologically confirmed thyroid cancer compared to those with benign histology. The quantitative and qualitative analysis of circulating $BRAF^{V600E}$ in the same patients before and after treatment (surgery and possible RAI) strongly indicates a causal relationship between circulating $BRAF^{V600E}$ and the presence of thyroid cancer.

The clinical performance of circulating $BRAF^{V600E}$ in thyroid cancer has, however, several intrinsic limits. Based on data obtained in tissue samples, $BRAF^{V600E}$ DNA occurs in 25–85% of thyroid cancer, depending on the patient population and the histological subtype (20), and this will account for false-negative samples. Furthermore, $BRAF^{V600E}$ somatic mutation is also present in nonthyroid cancer, such as melanoma and colorectal adenocarcinoma, and circulating $BRAF^{V600E}$ might in these cases be a possible source of false-positives for thyroid cancer (19). However, it is worthy of notice that we demonstrated a significant correlation between plasma and tissue *BRAF* status in a subset of patients. Regarding discordant cases, $BRAF^{V600E}$-mutated alleles detected in plasma of patients with wild-type primary tumor may have been derived from a portion of the biopsied tumor that was not used for the tissue-based PCR, as already reported by some other authors (33). Postsurgery Tg levels in PTC patients of this group were always undetectable and therefore did not provide additional information.

Keeping this information in mind, we demonstrated that circulating $BRAF^{V600E}$ has an acceptable negative predicting value for PTC in the Thy 3 cytology group, and its detection may be associated to the molecular profile in nodular tissues to avoid unnecessary surgery. In the diagnostic setting, the evaluation of circulating $BRAF^{V600E}$ may be useful when multiple nodules are present. In these patients a circulating marker, such as $BRAF^{V600E}$, might identify thyroid cancer within the whole pool of nodules, whereas FNA will provide information only for the sampled nodules, usually no more than 2.

After surgery or surgery plus RAI therapy, 5 patients out of 17 remained positive for circulating $BRAF^{V600E}$. Among these patients, 2 showed a marked positivity, and the persistence of disease was suspected. These results seem to indicate that circulating $BRAF^{V600E}$ could be associated with the persistence of disease and are in line with the data obtained by Cradic et al (22).

It is worthy of note that the 2 patients in whom circulating $BRAF^{V600E}$ was not detected before treatment remained negative also at the post-treatment sampling.

The only marker currently used in the follow-up of PTC is Tg, which is extremely sensitive and reliable (24) but may be less informative in some subsets of patients. This is particularly true when technical pitfalls may interfere with Tg assays. This is the case of anti-Tg antibody positivity that occurs in 25% of patients (25).

Other settings that reduce the predictive values of circulating Tg are related to the type of treatment. In fact, Tg is a marker of thyroid tissue but is not specific for neoplastic tissue, and its power in predicting cancer recurrence is limited when normal thyroid tissue is spared by surgical therapy. In addition, recent guidelines limited indications to RAI treatment (5, 6), and in these patients the presence of normal thyroid tissue belittles the use of Tg. In all these cases, circulating $BRAF^{V600E}$ may become an additional tool to monitor patients that have suffered with PTC.

Few other circulating markers have been proposed so far for the follow-up of PTC (15–17). Among these, the detection of circulating TSH receptor mRNA has provided extensive data. However, TSH receptor is, like Tg, a marker of normal thyroid tissue, and it will be very interesting to assess whether there is a correlation between circulating $BRAF^{V600E}$ and TSH receptor mRNA and whether the combination of these 2 biomolecular markers will have a synergistic effect.

Finally, circulating $BRAF^{V600E}$ could be useful in selecting patients with advanced thyroid carcinoma to be treated with newly developed small molecule inhibitors of the activity of $BRAF^{V600E}$ (eg, PLX4720, PLX4032 and sorafenib) that are promising approaches to the treatment of these patients (34–36).

When analyzed with respect to histological subtypes, we found that circulating $BRAF^{V600E}$ was more frequent in conventional papillary carcinomas (72%) than in the follicular variant (54%). These results are in agreement with the data reported in tissues (37).

In summary, the results of the present study provide encouraging data supporting the possibility to take advantage of circulating $BRAF^{V600E}$ in the management of PTC. However, additional studies will be required before circulating $BRAF^{V600E}$ could be widely used in clinical settings.

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Disclosure Summary: The authors have nothing to disclose.

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