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Circulating *BRAF*^{V600E} Levels Correlate with Treatment in Patients with Thyroid Carcinoma

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Background: *BRAF*^{V600E} is the most common mutation in papillary thyroid carcinoma (PTC) and can be associated with aggressive disease. Previously, a highly sensitive blood RNA-based *BRAF*^{V600E} assay was reported. The objective of this study was to assess the correlation of *BRAF*^{V600E} circulating tumor RNA levels with surgical and medical treatment.
Methods: Circulating *BRAF*^{V600E} levels were assessed in (i) a murine model of undifferentiated (anaplastic) thyroid carcinoma with known *BRAF*^{V600E} mutation undergoing *BRAF*^{V600E}-inhibitor (*BRAFi*) treatment, and (ii) in 111 patients enrolled prior to thyroidectomy ($n=86$) or treatment of advanced recurrent or metastatic PTC ($n=25$). Blood samples were drawn for *BRAF*^{V600E} analysis before and after treatment. Testing characteristics were assessed and positivity criteria optimized. Changes in blood *BRAF*^{V600E} values were assessed and compared to clinical characteristics and response to therapy.
Results: In a murine model of anaplastic thyroid carcinoma with *BRAF*^{V600E} mutation, blood *BRAF*^{V600E} RNA correlated with tumor volume in animals treated with *BRAFi*. In tissue *BRAF*^{V600E}-positive ($n=36$) patients undergoing initial surgery for PTC, blood *BRAF*^{V600E} levels declined postoperatively (median 370.0–178.5 fg/ng; $p=0.002$). In four patients with metastatic or poorly differentiated thyroid carcinoma receiving targeted therapies, blood *BRAF*^{V600E} declined following therapy and corresponded with radiographic evidence of partial response or stable disease.
Conclusions: This study shows the correlation of blood *BRAF*^{V600E} levels in response to treatment in both an established animal model of thyroid cancer and in patients with *BRAF*^{V600E}-positive tumors with all stages of disease. This assay represents an alternative biomarker in patients with positive thyroglobulin antibodies, and tumors, which do not express thyroglobulin.

Keywords: papillary thyroid cancer, thyroid cancer, *BRAF*^{V600E}, biomarker, circulation tumor cells

Introduction

Thyroid cancer has been increasing in incidence greater than any other cancer and is expected to be the third most common cancer in women by 2019 (1,2). Long-term disease-specific survival is excellent for most patients in this younger-skewing population, leading to an escalating prevalence of disease. While initially this increase in incidence was entirely attributed to increased diagnosis of small indolent tumors, a recent report shows increases in higher risk tumors as well (3). Acknowledgment of overdiagnosis of indolent disease and report of active surveillance protocols in Japan, among other observations, has led to a general shift in recommendations for less aggressive treatment of papillary thyroid carcinoma (PTC) (4–6). Improved risk stratification is highlighted as an important focus of thyroid cancer research (5).

Most risk-stratification algorithms were designed to predict mortality and do not integrate mutational status. The 2009 American Thyroid Association (ATA) guidelines for the management of differentiated thyroid cancer introduced a three-tiered risk of persistent/recurrent disease following initial treatment, which was validated in a number of countries (7). In the 2015 revision of the ATA guidelines, the authors include *BRAF*^{V600E} as a potential additional prognostic variable (5). *BRAF*^{V600E} causes constitutive activation

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of the MAPK pathway and is present in more than one half of PTCs and in 5–20% of patients with poorly differentiated and anaplastic thyroid carcinomas (ATC) (8,9). The proportion of *BRAF*^{V600E}-positive PTC is reportedly increasing (10). In thyroid cancer patients, this mutation is highly specific for PTC. Commercial DNA and gene expression profile assays performed on thyroid fine-needle aspirates (FNA) are highly predictive of malignancy when the *BRAF*^{V600E} DNA mutation is present (11–13). Currently, assessment of the thyroid tumor tissue for this mutation requires obtaining genetic material for mutational profiling through FNA or tissue for immunohistochemical staining with specific anti-*BRAF*^{V600E} antibodies (14). Traditional tissue assays are considered less sensitive because of the potential for background tissue contamination and sampling error (i.e., testing an adjacent *BRAF*^{wt} nodule) (5).

Knowing the mutation status of each tumor is important, since the mutation is associated with aggressive pathological features, local tumor recurrence, loss of radioactive iodine avidity (RAI), and increased disease-specific mortality (15–21). Even in papillary thyroid microcarcinomas (<1 cm), aggressive features and a higher risk of recurrence are observed in patients with *BRAF*^{V600E} tumors (22,23). While prophylactic central cervical lymph node dissection in patients with cytological *BRAF*^{V600E} tumors is controversial, some experts recommend using *BRAF*^{V600E} status to guide extent of initial surgery and use of RAI (24–27).

Recently, the development and feasibility of a highly sensitive blood RNA-based *BRAF*^{V600E} assay in patients with thyroid disease was reported (28). Circulating *BRAF*^{V600E} was able to be detected in the blood of thyroid cancer patients, and a good correlation was found with conventional tissue methods. The correlation of response to treatment in patients with melanoma undergoing *BRAF*^{V600E}-inhibitor (*BRAFi*) therapy has also been reported (5,29–32). In patients with thyroid cancer, a liquid biopsy that can accurately measure circulating *BRAF*^{V600E} levels would be beneficial at multiple levels: (i) in patients with tumor recurrences, where tissue is not easily accessible; (ii) an alternative biomarker for surveillance of those patients with thyroglobulin antibodies (TgAb); (iii) a biomarker for advanced thyroid cancer patients undergoing *BRAF*^{V600E}-targeted therapies; and (iv) an alternative to Tg in patients undergoing thyroid lobectomy alone for lower-risk PTC, since the remnant gland is left *in situ* (5,30–32). Compared to FNA, a routine blood draw is less expensive and less invasive. In addition, the FNA can only be done on one lesion at a time.

This work further characterized the RNA-based blood assay to see if it would allow for serial, quantitative analysis correlating with effects of treatments such as surgery or targeted therapies. In addition, *BRAF*^{V600E} levels were analyzed in a murine model of undifferentiated (anaplastic) thyroid carcinoma harboring the *BRAF*^{V600E} mutation during treatment with *BRAF*^{V600E} inhibitors. It was hypothesized that blood *BRAF*^{V600E} levels would correlate with response to treatment. Serial blood *BRAF*^{V600E} levels could provide an inexpensive, safe, and simple mechanism for risk stratification and surveillance in the 50% of patients with PTC with *BRAF*^{V600E} mutation, offer an alternative biomarker in in patients where Tg levels are not informative, and provide longitudinal assessment of treatment response.

Materials and Methods

Orthotopic murine model of human ATC

All animal work was done in the animal facility at Massachusetts General Hospital (Boston, MA) in accordance with federal, local, and institutional guidelines. Human ATC cells (8505c; *BRAF*$^{V600E/-}$, TP53$^{R248G/-}$; Deutsche Sammlung von Mikroorganismen und Zellkulturen) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 37°C 5% CO_2 incubator. An orthotopic murine model of *BRAF*^{V600E}-mutant ATC was chosen in order to provide proof-of-principle of the feasibility of detecting plasma *BRAF*^{V600E} RNA and the ability to correlate plasma levels with tumor volume. The 8505c human ATC orthotopic severe combined immunodeficiency (SCID) model progresses at a much faster rate compared to PTC models, which lends to improved ease of use. Human ATC cells were injected into the thyroid of 33 ten-week-old female SCID mice, as previously described (33). Briefly, the mice were anesthetized, the thyroid gland exposed, and 10^6 8505c cells were injected into the left thyroid lobe with a 27-gauge needle. The right thyroid lobe was not manipulated to serve as an internal control. Mice were randomized to either a control diet (Research Diets, Inc.) or 418 mg/kg *BRAFi* (PLX4720)-embedded chow (Research Diets, Inc.) *ad libitum*, starting two weeks after injection (34). Three mice were euthanized weekly for assessment of tumor volume (volume = [length × width × depth]/2), and blood was obtained by cardiopuncture and pooled to yield sufficient quantity. The blood *BRAF*^{V600E} level was quantified, as previously described and below (29).

Patient selection

Under approval by the Partners Human Research Committee Institutional Review Board at the Massachusetts General Hospital, patients with benign and malignant thyroid disorders undergoing initial curative surgery or treatment of recurrent disease were enrolled between September 2013 and September 2015. After informed consent, a 5 mL sample of peripheral blood was obtained from each patient before and after surgery or, in the cases of iodine-refractory metastatic disease, during treatment with targeted chemotherapies. The post-treatment blood was drawn at the time of the subsequent clinic visit, the vast majority being at the postoperative visit (median 17.5 days; interquartile range (IQR) 13.0 to 30.3 days).

Blood BRAF^{V600E} *assay protocol*

The assay was previously described in detail (28,29). Briefly, peripheral blood lymphocytes were isolated by Ficoll density centrifugation from each blood sample and stored in freezing medium. RNA was isolated by the Trizol method (Invitrogen, Grand Island, NY) and (50–100 ng) reverse transcribed to cDNA by standard methods, normalized in quantity with 18S RNA, and amplified. After DNA cleanup, wild-type *BRAF* (*BRAF*^{wt}) was digested with TSPR1 (restriction site = NNCASTGNN; New England Biolabs, Beverly, MA) to reduce contamination by normal *BRAF*^{V600E} from surrounding normal tissue in the blood samples. The product was then subjected to a second round of polymerase chain reaction (PCR) and *BRAF*^{wt} digestion. To favor the

mutant further over the wild-type product, a 33-fold excess of the reverse (common sequence in mutant and wild-type) to forward (exact match for mutant and one base mismatch for wild-type sequences) primers were used in the final real-time PCR assay for $BRAF^{V600E}$. Oligonucleotides were custom synthesized from Invitrogen (Carlsbad, CA) and Sigma–Aldrich (St. Louis, MO). Purified $BRAF^{V600E}$ first-round PCR product with a known concentration was also run through the assay and was used to create a standard curve.

It was previously established that the assay can detect as low as 1 pg of $BRAF^{V600E}$ and has a 1000-fold increased sensitivity compared to the $BRAF^{wt}$ (28). This assay was used for the orthotopic murine model (discussed above), reported in pictograms, with a positivity criterion of 4.8 pg. The 18S reverse transcription PCR assay is now run in the presence of known amounts of purified RNA in order to generate a standard curve (input RNA in nanograms). Going forward, all new data generated by this assay will be reported as femtograms of $BRAF^{V600E}$/nanogram of RNA.

Tissue-based $BRAF^{V600E}$ *analysis*

Tissue-based $BRAF^{V600E}$ analysis has been previously described (28). Briefly, patients meeting inclusion criteria and with tissue available had $BRAF^{V600E}$ mutational analysis as part of standard clinical care either via SNaPshot (Massachusetts General Hospital Cancer Center Translational Research Laboratory) or immunohistochemistry was done using a $BRAF^{V600E}$ monoclonal antibody with 97% correlation with SNaPshot molecular testing (98% sensitivity and 97% specificity) (35). For PTC cases in which mutational status was not obtained clinically, $BRAF^{V600E}$ mutation was sequenced from formalin-fixed, paraffin-embedded (FFPE) tissue blocks, as previously described.

Tg assay

A commercial assay (Mayo Medical Laboratories New England, MA) was used to determine Tg levels in all patients, as per standard clinical practice.

Statistical analysis

Clinical variables were chosen based on established demographic and pathological risk factors for decreased thyroid cancer-free survival. The American Joint Committee on Cancer TNM (AJCC TNM) stage was determined for each patient. For comparisons between $BRAF^{V600E}$ and wild-type groups, pathological variables were only considered present if they were specifically described in the final pathology report (as per convention). Univariate comparisons of categorical variables were analyzed using Fisher's exact test, and continuous variables were assessed by Student's *t*-test or Wilcoxon's rank-sum test for nonparametric data. Correlations of postsurgical blood $BRAF^{V600E}$ expression levels according to mutational status and demographic and pathological characteristics were based on linear regression models. A p-value of <0.05 was considered statistically significant. Using tissue results as the gold standard, likelihood ratios (sensitivity/1 – specificity) were calculated, and a receiver operating characteristic curve was produced. An *a priori* decision was made to maximize specificity for determining the positivity criterion. Blood $BRAF^{V600E}$ levels before and after surgery were compared, and Wilcoxon's signed rank test was carried out to assess the non-normal blood $BRAF^{V600E}$ level change before and after surgery for tissue $BRAF^{V600E}$-positive and tissue $BRAF^{wt}$ patients, respectively.

Results

Circulating $BRAF^{V600E}$ *levels correlate with tumor response after treatment with* BRAFi *in an orthotopic murine model of ATC*

The previously described immunocompromised orthotopic mouse model of ATC was utilized to study the correlation between $BRAF^{V600E}$ tumor growth and $BRAF^{V600E}$ levels both before and following treatment with the selective *BRAFi* (PLX4720) (33). This model results in tumor growth with extrathyroidal extension over several weeks and cervical lymph node and pulmonary metastases; control animals have a life expectancy of up to five weeks.

A total of 33 mice had orthotopic injection of 8505c cells (Fig. 1). Three mice were euthanized weekly to evaluate mean tumor volumes, and their blood was pooled to obtain sufficient peripheral blood leukocytes for RNA-based $BRAF^{V600E}$ analysis. Two days after implantation of tumor, mouse blood $BRAF^{V600E}$ levels were 16.17 pg and steadily rose such that at two weeks post implantation, mice had a peak $BRAF^{V600E}$ RNA level of 40.8 pg in the pooled blood and a mean tumor volume of $12.0 \pm 0.6\,mm^3$. Subsequently, mice were randomized to either control chow or treatment with *BRAFi* embedded chow at a standard dose (418 mg/kg *BRAFi*). Over the next set of measurements, tumor volume rose steadily to a peak of $118.6 \pm 26.1\,mm^3$ at four weeks in the control group, consistent with previously reported data. Blood $BRAF^{V600E}$ levels remained elevated at each weekly time point (34). During the third week post implantation, the $BRAF^{V600E}$ blood level dipped slightly to 22.01 pg and remained stable at four weeks. The control mice had to be euthanized at the beginning of week 5 post implantation in order to meet humane endpoints. In the *BRAFi*-treated group, tumor volume declined to $7.11 \pm 1.0\,mm^3$ and $2.53 \pm 2.7\,mm^3$ at weeks 3 and 4, respectively. Tumors subsequently became and remained undetectable. Mean blood $BRAF^{V600E}$ RNA declined in tandem with tumor volume to 2.1 pg and 4.0 pg at weeks 3 and 4, respectively, and remained between 1.5 pg and 2.8 pg through week 8 of the study.

Patient cohort characteristics

The goal was to analyze this assay in a variety of thyroid cancer patients to understand assay utility fully. The study population included 111 patients who had their blood $BRAF^{V600E}$ level measured before and after surgery or adjuvant therapy (Fig. 2). Of the 111 patients, 25 were undergoing advanced thyroid cancer therapy, including seven PTC patients who had surgery for recurrent local disease and 18 patients with RAI-resistant differentiated, poorly differentiated, or ATC undergoing active surveillance, or systemic therapy with small molecule inhibitors or, in the case of ATC, cytotoxic chemotherapy and radiotherapy. Fifty-nine patients underwent initial surgical treatment with curative intent for PTC, 51 (94.4%) of whom underwent at least total thyroidectomy. In this group, 36 patients had tumor tissue harboring

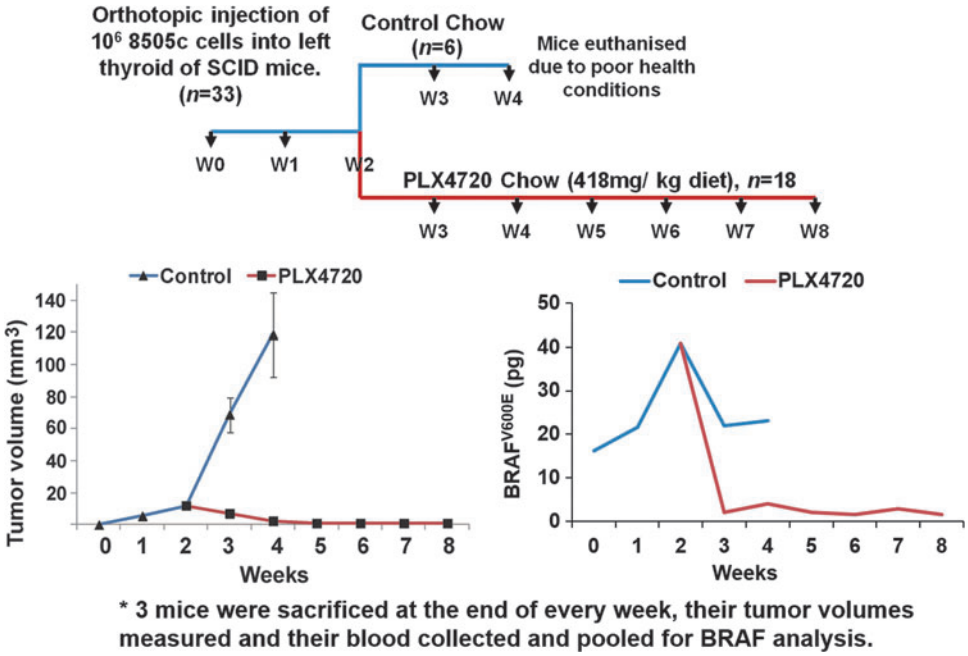

FIG. 1. *BRAF*^{V600E}-mutated thyroid tumors grow in the first two weeks after orthotopic tumor implantation. Blood *BRAF*^{V600E} levels also increase. Treatment with *BRAFi* (PLX4720) prevents tumor progression and dramatically reduces blood *BRAF*^{V600E} levels.

the *BRAF*^{V600E} mutation, whereas 18 patients had tumor tissue with *BRAF*^{wt}. In five patients, the assay failed ($n=1$) or *BRAF*^{V600E} testing was not performed. An additional 27 patients were recruited as controls, and their surgical pathology diagnoses were multinodular goiter ($n=23$) and follicular adenoma ($n=4$). None of the patients had known melanoma or colorectal adenocarcinoma. Patients with cancer had AJCC TNM stages I–IV (Table 1).

Testing characteristics

To compare the blood *BRAF*^{V600E} assay results with the conventional tissue testing, the results of the optimized blood assay were compared to conventional tissue mutational testing using the group of patients undergoing initial surgery for curative intent in which tissue genotyping was available. Using the tissue *BRAF*^{V600E} result as the gold standard, likelihood ratios were calculated and a receiver operating characteristic curve for the blood assay predicting tissue *BRAF*^{V600E} mutational status was constructed (Fig. 3). The area under the curve (AUC) was 0.68, indicating moderate correlation of tests assuming the tissue results are true. Using maximum likelihood ratio for a positive test (LR^+) as the optimal cutoff, the threshold for blood *BRAF*^{V600E} level is 140–145 fg/ng RNA.

Blood BRAF^{V600E} *levels decline following initial surgical treatment for PTC*

Preoperative blood *BRAF*^{V600E} levels were assessed for association with clinical and pathological characteristics. On multivariable linear regression, adjusting for sex, age, and tumor volume, only extrathyroidal extension was correlated with the preoperative blood *BRAF*^{V600E} level ($p=0.03$). The blood *BRAF*^{V600E} levels were compared before and after initial surgical treatment for PTC patients stratified by their tissue *BRAF*^{V600E} status (Fig. 4). For tissue *BRAF*^{V600E}-positive patients ($n=36$), the median blood *BRAF*^{V600E} level dropped from 370.0 fg/ng RNA to 178.5 fg/ng RNA ($p=0.002$). For tissue *BRAF*^{wt} ($n=18$) and for non-PTC controls with tissue *BRAF*^{wt} ($n=15$), the median blood *BRAF*^{V600E} level did not change significantly with surgery ($p=0.70$ and $p=0.69$, respectively). Three of these patients for whom blood *BRAF*^{V600E} levels were available before and after initial surgery for curative intent had a structural recurrence requiring surgery during the study period, all of whom were tissue *BRAF*^{V600E} positive. Blood *BRAF*^{V600E} levels correlated with clinical disease status in the two patients with high preoperative levels. In one patient, the preoperative *BRAF*^{V600E} level of 3567 fg/ng RNA fell to139 fg/ng (Tg 8.8 ng/mL), below the established threshold, at six months postoperatively. With the development of recurrent clinical nodal disease six months later, the *BRAF*^{V600E} level had increased to 316 fg/ng RNA and correlated with an increase in the Tg level, indicating recurrence (Tg level 54 ng/mL). To see whether serum thyrotropin (TSH) levels influenced blood *BRAF*^{V600E} levels, the length of the time lapse between collection of the blood *BRAF*^{V600E} levels, tissue *BRAF*^{V600E} levels, and the recorded TSH were adjusted, and no significant association was found between blood *BRAF*^{V600E} levels and TSH levels following surgery (data not shown). The effect between postoperational serum *BRAF*^{V600E} level and the time between surgery and measurement was also tested (median 17.5 days; IQR 13.0 to 30.3 days), adjusting for preoperative blood *BRAF*^{V600E} levels and tissue *BRAF* status in a linear regression model, and there was no significant association ($p=0.328$).

Blood BRAF^{V600E} *levels before and after surgical treatment for locally recurrent PTC*

Seven patients in the cohort had remote thyroidectomy with or without cervical lymph node dissection. Five of the patients had tumor tissue positive for *BRAF*^{V600E}, one in the index tumor and four in the recurrent tumors. Blood

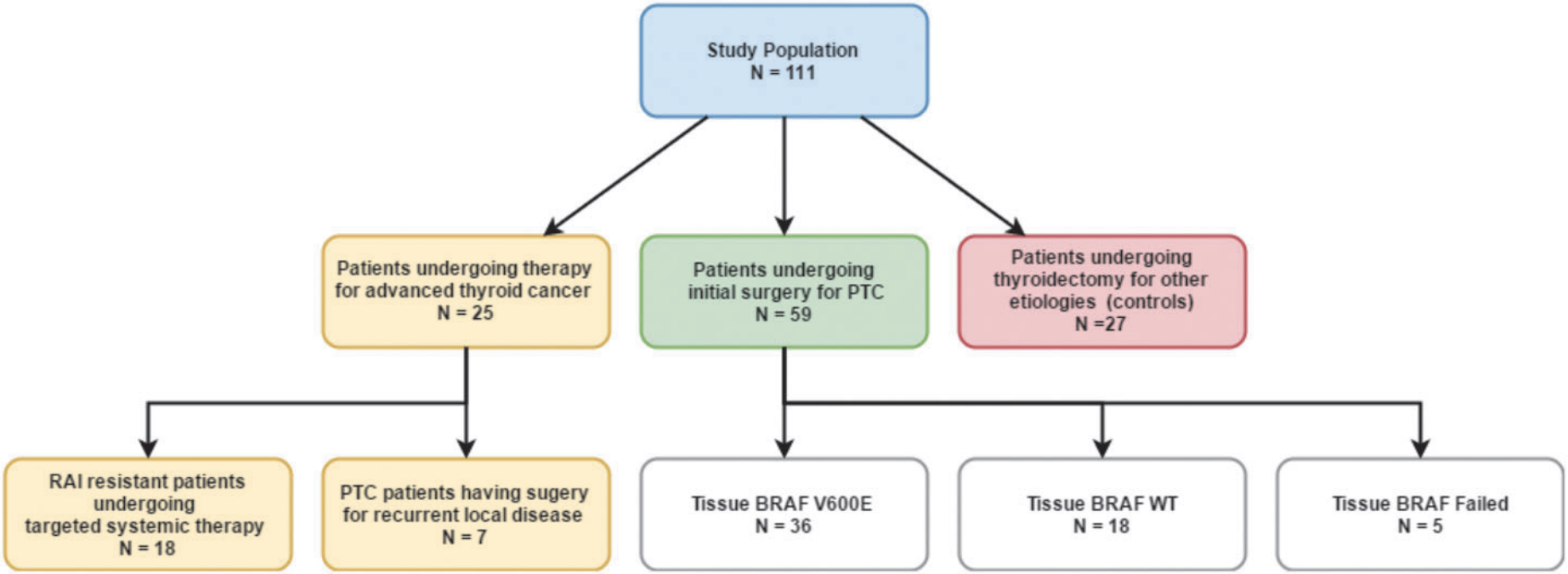

FIG. 2. Patient cohort and associated therapy.

TABLE 1. CHARACTERISTICS OF COHORT PATIENTS WHO UNDERWENT INITIAL SURGERY FOR CURATIVE TREATMENT FOR PTC

	All (n = *54*)	*Tissue* BRAF^{V600E} *positive* (n = *36*)	*Tissue* BRAF^{wt} (n = *18*)	p-*Value*
Demographics				
Female, *n* (%)	38 (70)	25 (69)	13 (72)	0.99
Age at surgery, *M* (±*SD*)	46.9 (15.1)	44.8 (13.5)	51.0 (17.5)	0.16
Primary tumor characteristics				
Tumor maximum diameter (mm), *M* (±*SD*)	1.9 (1.3)	1.9 (1.2)	1.8 (1.3)	0.92
Extrathyroidal extension, *n* (%)	19 (35)	14 (39)	5 (28)	0.55
Lymphovascular invasion, *n* (%)	27 (51)	21 (58)	6 (35)	0.15
Cervical lymph node status, n *(%)*				
Central lymph node metastases	23 (42.6)	18 (50.0)	5 (27.8)	0.15
Lateral lymph node metastases	11 (20.4)	8 (22.2)	3 (16.7)	0.73
AJCC TNM stage, n *(%)*				0.35
I	37 (69)	25 (69)	12 (67)	
II	4 (7)	1 (3)	3 (17)	
III	5 (9)	4 (11)	1 (6)	
IV	8 (15)	6 (17)	2 (11)	
Blood BRAF^{V600E} *level*				
Preoperative level (fg/ng RNA), median (IQR)	301.5 (136.3–608.0)	370.0 (203.3–686.5)	136.5 (85.3–354.0)	0.03
Postoperative level (fg/ng RNA), median (IQR)	177.5 (80.0–254.8)	178.5 (75.8–305.5)	158.0 (87.0–223.3)	0.73

PTC, papillary thyroid carcinoma; wt, wild type; *SD*, standard deviation; AJCC TNM, American Joint Committee of Cancer Tumor, Node, Metastasis; IQR, interquartile range.

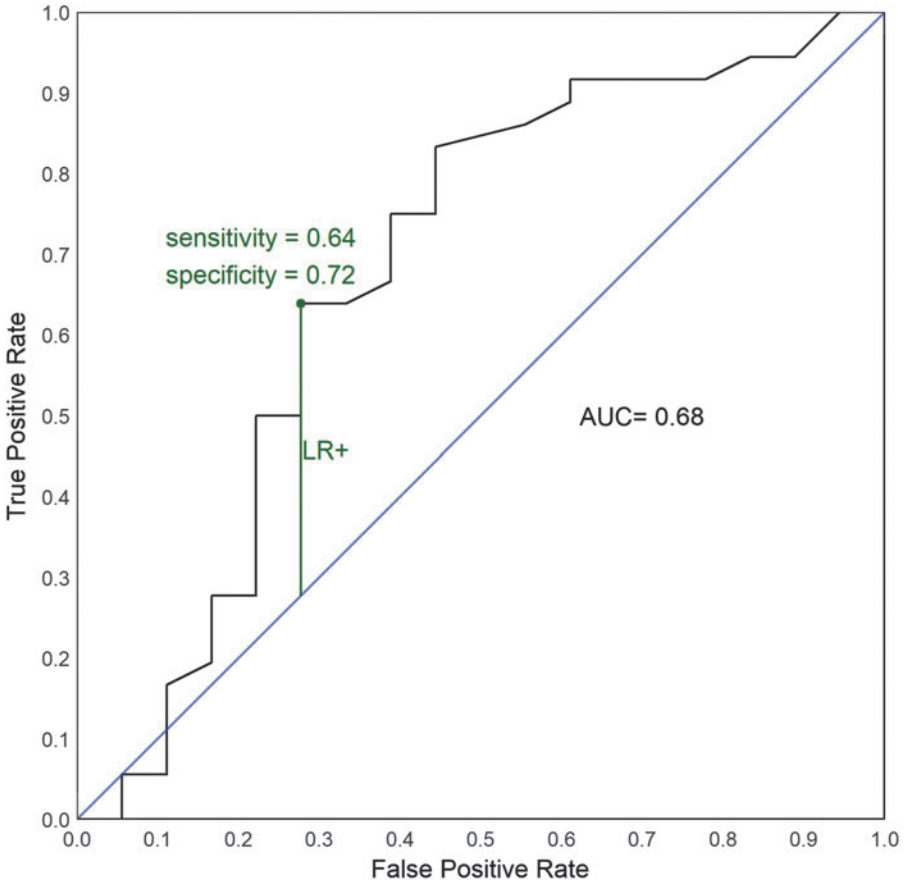

FIG. 3. Receiver operating characteristic curve based on 54 patients receiving initial papillary thyroid carcinoma therapy who have tissue *BRAF*^{V600E} status.

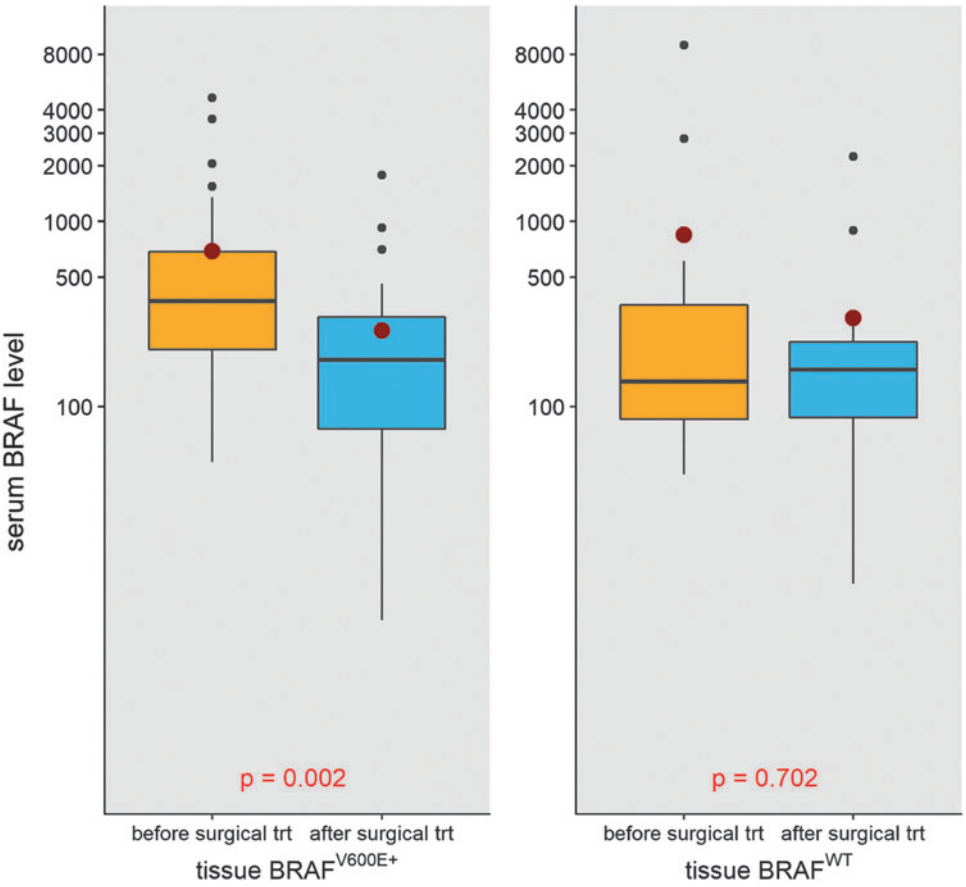

FIG. 4. Comparison of blood $BRAF^{V600E}$ level ($\log_{10}$ scale) before and after initial surgical treatment among tissue $BRAF^{V600E}$-positive and $BRAF^{WT}$ patients. The box plots measure 1st quartile −1.5 interquartile range (IQR), 1st quartile, median, 3rd quartile, 3rd quartile +1.5 IQR from bottom to top.

$BRAF^{V600E}$ was obtained before and after surgery to characterize recurrent local disease. Median blood $BRAF^{V600E}$ before and after salvage surgery was 319.0 fg/ng RNA (IQR 192.5–562.6 fg/ng) and 260 fg/ng RNA (IQR 109.0–491.0fg/ng). In one patient, clinical recurrence occurred one year following initial total thyroidectomy with central lymph node dissection for a tall-cell variant PTC with a small focus of ATC. The preoperative $BRAF^{V600E}$ level was 1193 fg/ng. While two weeks following salvage surgery this level failed to decline (2163 fg/ng), after external beam radiation, the level decreased to 598 fg/ng RNA. Six months following this, the level returned to 2104 fg/ng RNA, which was concerning for worsening disease. The patient has a new thyroid bed mass that is being observed (based on the patient's age and choice), which is consistent with the observed elevation in $BRAF^{V600E}$ levels. Throughout the clinical course, Tg levels have been very low to undetectable (0.1–0.3 ng/mL). Two other patients in this group had elevated TgAb.

Blood $BRAF^{V600E}$ *levels before and after initiation of targeted therapies*

Blood $BRAF^{V600E}$ samples were obtained from 18 patients with metastatic RAI-resistant papillary ($n=11$) poorly-differentiated/anaplastic ($n=5$), and Hürthle cell ($n=2$) thyroid carcinomas undergoing active surveillance, or receiving chemotherapy, radiotherapy, multikinase inhibitors, and/or MAP-kinase pathway-specific inhibitors. Fourteen of the patients had $BRAF^{V600E}$-mutant tumors, and all 18 had detectable levels of $BRAF^{V600E}$ in their blood (median 615 fg/ng; range 41–6552 fg/ng). Six patients (five $BRAF^{V600E}$-positive PTC and one with poorly differentiated thyroid carcinoma [$BRAF^{V600E}$ positive]) did not have an alternative means of biochemical monitoring due to lack of Tg production (Tg <1 ng/mL; $n=4$) or because of elevated TgAb ($n=2$). Five of these six patients without other means of biochemical monitoring had gross elevations in pretreatment blood $BRAF^{V600E}$ levels (range 552–6552 fg/ng) that dropped following systemic therapy initiation (range of $\log_2$ [fold change] of 0.76–4.91). Correlation between blood $BRAF^{V600E}$ and treatment response per RECIST v 1.1 was most notable in patients receiving $BRAF^{V600E}$ inhibitors alone or in combination with MEK inhibitors (Fig. 5). In this subset of four patients, blood $BRAF^{V600E}$ levels declined dramatically following initiation of therapy and corresponded with radiographic evidence of partial response or stable disease. In three of these patients, blood $BRAF^{V600E}$ increased from the nadir, one of whom developed progressive disease 13 months after this increase, while the two other patients continued to have stable disease.

Discussion

Thyroid cancer has been increasing in incidence more than any other cancer in the past few decades. While the majority of the increase is attributed to small, seemingly indolent cancers, a recent report also shows an increase in higher-staged tumors and disease-specific mortality (3). With recent

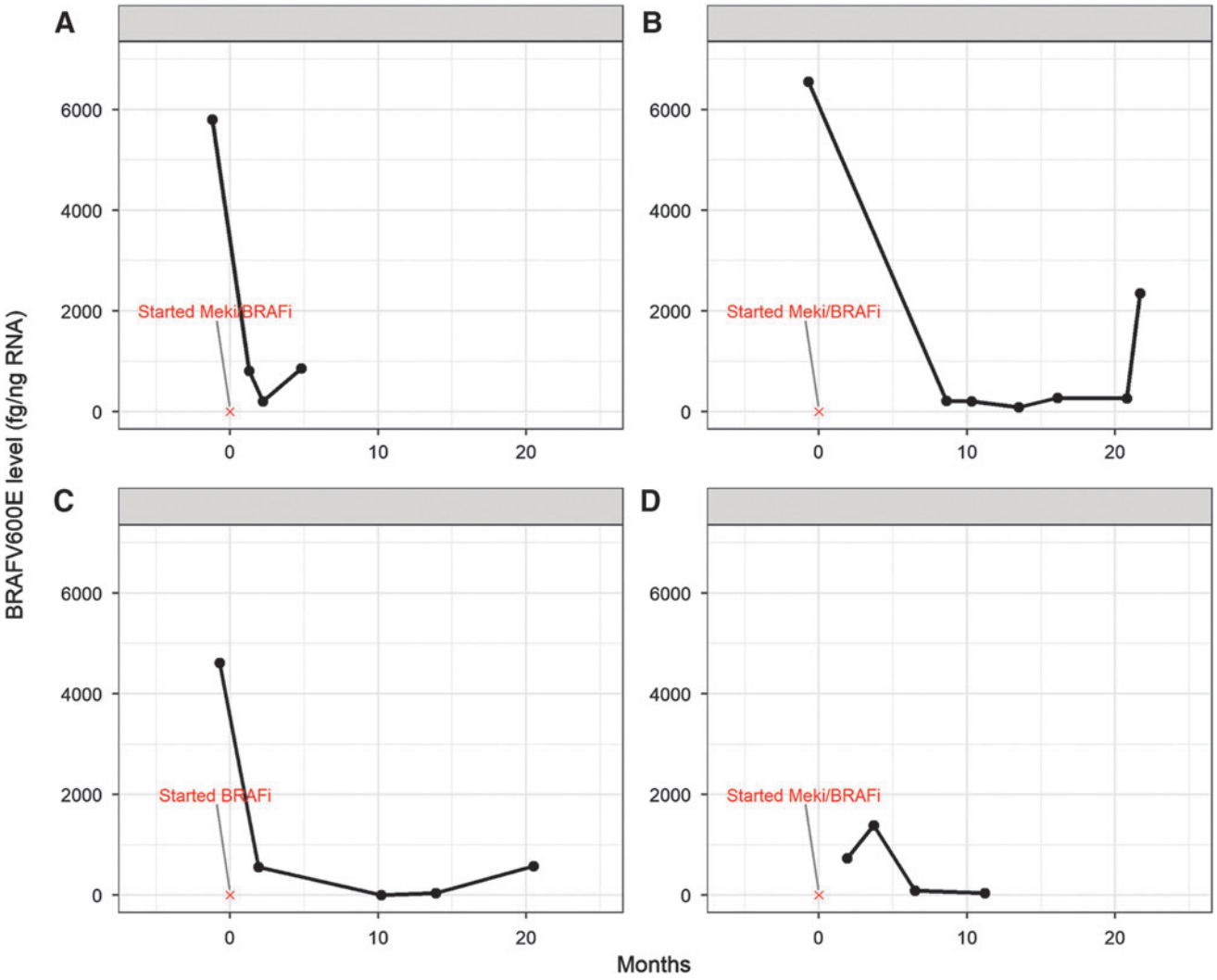

FIG. 5. Circulating levels of *BRAF*^{V600E} in four patients with radioiodine-refractory metastatic *BRAF*^{V600E}-positive thyroid cancer undergoing targeted therapy. *BRAFi*, dabrafenib or vemurafenib; MEKi, trametinib.

changes in guidelines toward less aggressive treatment, improved risk stratification for thyroid cancer patients is essential (5). Additionally, the standard circulating biomarker, Tg, is unreliable in the subset of patients who progress to aggressive disease and whose tumors no longer make Tg, as well as in patients with TgAb that interfere with measurement of Tg. Thus, alternative biomarkers are needed.

Approximately half of patients with PTC harbor a *BRAF*^{V600E} mutation, a proportion that may be growing, at least in North America, with the reclassification of *RAS*-mutant (*BRAF*^{WT}) noninvasive encapsulated follicular-variant PTC to noninvasive follicular thyroid neoplasm with papillary-like nuclear features (36). Large controlled studies have shown a correlation between this mutation and aggressive tumor characteristics, tumor recurrence, and overall survival particularly in the presence of other mutations such as *TERT* (15,17,18,37). Knowledge of *BRAF*^{V600E} status may be helpful in diagnosis (nearly 100% positive predictive value for PTC); determining extent of surgery (lobectomy vs. total thyroidectomy ± level VI cervical lymph node dissection), use of RAI, and frequency and intensity of follow-up; as an alternative to Tg in Tg-negative or TgAb-positive patients; and to assess response to *BRAFi* therapy in patients with advanced disease. Conventionally, cytology or surgical pathology specimens have been utilized to ascertain *BRAF*^{V600E} mutational status. The feasibility of a sensitive RNA-based blood assay to detect and serially monitor *BRAF*^{V600E} levels in patients with PTC has been published previously (28).

A key potential advantage of the assay is to serve as an alternative biomarker in patients with anti-TgAb, present in a significant proportion of PTC patients, and in the subset of patients whose tumors stop producing Tg because of disease dedifferentiation (38). Indeed, 12/59 (20%) patients undergoing initial surgery for PTC in this study had TgAb noted postoperatively, making Tg levels useless. Of these 12, nine were positive for *BRAF*^{V600E} on tissue testing. Indeed, in 6/18 patients with advanced thyroid cancer, the *BRAF*^{V600E} levels were elevated in the setting of very low or absent Tg <1.0 ng/mL and/or anti-TgAb ($n = 2$). Moreover, in the small but substantial group of patients destined to do poorly, there is an enrichment of *BRAF*^{V600E} mutations. Thus, the ability to

detect and follow $BRAF^{V600E}$ levels accurately over time as a biomarker for recurrence and response to *BRAFi* would be of great clinical value.

This work shows the correlation of blood $BRAF^{V600E}$ levels in response to treatment both in an established animal model of thyroid cancer and in $BRAF^{V600E}$ patients with all stages of disease undergoing treatment. In both the orthotopic murine model and in the patients, blood $BRAF^{V600E}$ levels were not associated with tumor size, TSH level, Tg levels, or time of blood draw. In fact, the only clinical or pathological factor correlating with $BRAF^{V600E}$ levels in the patient cohort was extrathyroidal extension. It was found in general that blood $BRAF^{V600E}$ levels were detectable in all stages of disease and responded to both surgical therapy and treatment with systemic targeted therapy.

It was observed that plasma $BRAF^{V600E}$ RNA levels spontaneously dropped in control mice that did not receive PLX4720 at week 3, albeit to a much lesser extent compared to mice that received treatment. The reasons for this decline (and subsequent rise) are unclear but may be due to the complex dynamic relationship between circulating tumor cells/nucleic acids and the originating tumor volume. In the 8505C orthotopic SCID ATC model, weeks 2–3 coincide with the exponential growth of thyroid tumor volume (in this experiment, mean tumor volume increased from 12.0 mm^3 to 68.9 mm^3) and the emergence of pulmonary micrometastases. In a review of the primary data, the subsequent rise in *BRAF*-mutant RNA at week 4 is largely driven by higher levels of the fourth mouse with a small tumor whose blood was analyzed separately. This was included at the four-week time point by taking a mean with a weighted mean of the pooled mice ($n = 3$). If this mouse is excluded from the analysis, the decline continues to occur. The outlier was included for completeness. Although not specifically shown for circulating tumor RNA, a spontaneous decrease in circulating tumor cells has been reported in the literature, especially during a period of rapid tumor growth following a period of slower growth (39–41). A spontaneous decline and then subsequent rise has also been reported, especially for larger tumors. There are several postulated mechanisms for this phenomenon that remain unproven. One such mechanism includes the activation of host immunity by increasing single or clustered circulating tumor cells and subsequent enhanced clearance, although this immune activation is not enough to penetrate and/or clear the primary solid tumor or metastases, which are associated with an immunosuppressive microenvironment. While one might expect this phenomenon to be dampened in SCID mice, they do retain functional phagocytes such as neutrophils and monocytes/macrophages, which may be contributory to clearance. Additionally, rapid tumor growth may be associated with changes in tumor vessel morphology and function and tumor cell apoptosis/necroptosis; it is conceivable that these variables might decrease the efflux of circulating tumor cells.

Limitations of our study are acknowledged. There is some baseline signal in both $BRAF^{wt}$ patients and patients undergoing thyroidectomy for benign disease. Using tissue $BRAF^{V600E}$ status of the dominant tumor as the reference may be a source of potential error, given that PTC is commonly multifocal, with some carcinomas harboring $BRAF^{V600E}$ mutations and others that do not. It is possible that the larger, dominant nodule undergoing tissue testing may be $BRAF^{wt}$ while other, potentially smaller, carcinomas (which may shed $BRAF^{V600E}$ into the blood) may not have been tested. Another possible explanation is that there is a small volume of *BRAF*-mutant cells in the thyroid neoplasm that was not identified, since it was below the level of detection of the *BRAF* tissue testing. The blood test may be more sensitive than tissue testing. However, this is impossible to prove without testing at the tissue level (although testing at the tissue level may be limited by heterogeneity between different lesions, as discussed above). Additionally, the possibility cannot be entirely excluded that detectable circulating levels of $BRAF^{V600E}$ are from an undiagnosed $BRAF^{V600E}$ mutant malignancy (e.g., melanoma or colorectal adenocarcinoma), although there was no clinical documentation of such in the cohort. None of these patients has declared themselves with a secondary malignancy thus far, although small PTC can be indolent. Lastly, it is also possible that there is a false-positivity rate or noise with the assay at low or absent $BRAF^{V600E}$ concentrations that will not be overcome until technical and analytical validation is formally completed, which is the subject of ongoing work. As with other biochemical assays, a functional sensitivity threshold will need to be established. The change from before to after treatment will likely be the most useful metric to follow.

Recently, several commercial $BRAF^{V600E}$ assays, originally designed for and validated on FFPE samples, have been investigated for use with circulating tumor DNA isolated from the blood of patients with melanoma, including the PrimePCR™ ddPCR™ $BRAF^{V600E}$ pV600E Mutation Assay (Bio-Rad, Hercules, CA), the therascreen CRGQ PCR Kit (Qiagen, Hilden, Germany), the Idylla™ $BRAF^{V600E}$ mutation test (Biocartis, Mechelen, Belgium), the PNA Clamp™ $BRAF^{V600E}$ Mutation Detection Kit (Panagene, Daejeon, Korea), and the ct$BRAF^{V600E}$ Mutation Detection Kit (Entrogen, Woodland Hills, CA) (42–48). Kim *et al*. (45) were the first to adopt the use of the TaqMan® (castPCR™) $BRAF^{V600E}$ Mutation Detection Assay (Life Technologies, Carlsbad, CA) in the plasma of patients with PTC. The present assay detects $BRAF^{V600E}$ amid excess wild-type *BRAF* with high sensitivity (28,29). The use of RNA with exponentially higher copy numbers of $BRAF^{V600E}$ mRNA as well as digestion of $BRAF^{wt}$ enhances the sensitivity of the assay, which is a potential advantage over the DNA-based techniques described above.

Blood-based $BRAF^{V600E}$ detection has several clinical applications. Unlike tissue mutational testing, in which results are qualitative and binary, this assay allows quantitative, serial measurements of circulating $BRAF^{V600E}$, and levels are responsive to treatment. The ability to test circulating $BRAF^{V600E}$ levels during treatment with *BRAFi* and other targeted therapies to assess response to treatment and prediction of re-differentiation would be of great value in guiding therapy. Recent work has shown the *BRAFi* (dabrafenib) treatment in RAI-refractory patients can lead to re-differentiation and subsequent RAI uptake in some tumor lesions (32). Partial response was also seen in a Phase II trial with the *BRAFi* vemurafenib in 10/26 patients with metastatic or unresectable RAI-refractory PTC (49). More recent work in two other Phase II trials using combinations of *BRAFi* and *MEKi* has shown high response rates in $BRAF^{V600E}$ RAI refractory PTC and $BRAF^{V600E}$ ATC patients (50,51), and one Phase I trial of dabrafenib and LAP

was well tolerated in *BRAF*^{V600E} thyroid cancer patients (52). The ability to test circulating *BRAF*^{V600E} levels during treatment with *BRAFi* to assess response to treatment (surgical, RAI, and systemic therapy) and as an early marker of recurrence could be of great value.

In summary, this study reports the correlation of RNA blood *BRAF*^{V600E} levels with surgical and medical management of patients with PTC. This builds on prior work showing that the assay has good correlation (AUC 0.71) with tissue *BRAF*^{V600E} testing (28). In a murine model of *BRAF*^{V600E}-mutant ATC, blood *BRAF*^{V600E} levels correlate with tumor volume in animals and correlate with clinical response to *BRAFi*. The study also reports a significant decline in levels following initial surgery for curative intent. While it is challenging to make conclusions in the heterogeneous population of patients with metastatic or poorly differentiated thyroid carcinomas receiving targeted therapies, a subset of patients was found who showed a decline in blood *BRAF*^{V600E} levels following therapy that corresponded with radiographic evidence of partial response or stable disease. It is possible that this blood assay may work best for patients with *BRAF*^{V600E}-mutated tumors who have high baseline levels and are undergoing specific targeted therapies that work on reducing MAPK signaling. This assay represents an alternative biomarker in patients with anti-TgAb, and tumors, which do not express Tg. This is important, since no other biomarker exists for this group of patients and standard radiologic means of measuring tumor responses such as RECIST are less reliable in thyroid cancer. Finding a reliable assay that can provide early information into the therapeutic effect at the tumor cell level by measuring the RNA expression of the tumor cells in the blood can be an important tool that the medical oncologists can use to gauge a response to a targeted therapy.

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References

1. Surveillance, Epidemiology, and End Results. Available at: http://seer.cancer.gov/ (accessed December 1, 2016).
2. Davies L, Morris LG, Haymart M, Chen AY, Goldenberg D, Morris J, Ogilvie JB, Terris DJ, Netterville J, Wong RJ, Randolph G; AACE Endocrine Surgery Scientific Committee 2015 American Association of Clinical Endocrinologists and American College of Endocrinology Disease state clinical review: the increasing incidence of thyroid cancer. Endocr Pract **21:**686–696.
3. Lim H, Devesa SS, Sosa JA, Check D, Kitahara CM 2017 Trends in thyroid cancer incidence and mortality in the United States, 1974–2013. JAMA **317:**1338–1348.
4. Haser GC, Tuttle RM, Su HK, Alon EE, Bergman D, Bernet V, Brett E, Cobin R, Dewey EH, Doherty G, Dos Reis LL, Harris J, Klopper J, Lee S, Levine RA, Lepore SJ, Likhterov I, Lupo MA, Machac J, Mandel SJ, Mechanick JI, Mehra S, Milas M, Orloff L, Randolph G, Revenson TA, Roberts KJ, Ross DS, Rowe ME, Smallridge R, Terris D, Tufano RP, Urken ML 2016 Active surveillance for papillary thyroid microcarcinoma: new challenges and opportunities for the health care system. Endocr Pract **22:** 602–611.
5. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, Steward DL, Tuttle RM, Wartofsky L 2016 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid **26:**1–133.
6. Ito Y, Oda H, Miyauchi A 2016 Insights and clinical questions about the active surveillance of low-risk papillary thyroid microcarcinomas [Review]. Endocr J **63:**323–328.
7. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, Mazzaferri EL, McIver B, Pacini F, Schlumberger M, Sherman SI, Steward DL, Tuttle RM 2009 Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid **19:**1167–1214.
8. Eloy C, Ferreira L, Salgado C, Soares P, Sobrinho-Simoes M 2015 Poorly differentiated and undifferentiated thyroid carcinomas. Turk Patoloji Derg **31:**48–59.
9. Penna GC, Vaisman F, Vaisman M, Sobrinho-Simoes M, Soares P 2016 Molecular markers involved in tumorigenesis of thyroid carcinoma: focus on aggressive histotypes. Cytogenet Genome Res **150:**194–207.
10. Kowalska A, Walczyk A, Kowalik A, Palyga I, Trybek T, Kopczynski J, Kajor M, Chrapek M, Pieciak L, Chlopek M, Gozdz S, Kaminski G 2016 Increase in papillary thyroid cancer incidence is accompanied by changes in the frequency of the *BRAF V600E* mutation: a single-institution study. Thyroid **26:**543–551.
11. Alexander EK, Schorr M, Klopper J, Kim C, Sipos J, Nabhan F, Parker C, Steward DL, Mandel SJ, Haugen BR 2014 Multicenter clinical experience with the Afirma gene expression classifier. J Clin Endocrinol Metab **99:**119–125.
12. Nam SY, Han BK, Ko EY, Kang SS, Hahn SY, Hwang JY, Nam MY, Kim JW, Chung JH, Oh YL, Shin JH 2010 *BRAF V600E* mutation analysis of thyroid nodules needle aspirates in relation to their ultrasongraphic classification: a potential guide for selection of samples for molecular analysis. Thyroid **20:**273–279.
13. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, Fagin JA, Falciglia M, Weber K, Nikiforova MN 2009 Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab **94:**2092–2098.
14. Dvorak K, Aggeler B, Palting J, McKelvie P, Ruszkiewicz A, Waring P 2014 Immunohistochemistry with the anti-*BRAF V600E* (VE1) antibody: impact of pre-analytical

conditions and concordance with DNA sequencing in colorectal and papillary thyroid carcinoma. Pathology **46:** 509–517.
15. Elisei R, Viola D, Torregrossa L, Giannini R, Romei C, Ugolini C, Molinaro E, Agate L, Biagini A, Lupi C, Valerio L, Materazzi G, Miccoli P, Piaggi P, Pinchera A, Vitti P, Basolo F 2012 The *BRAF(V600E)* mutation is an independent, poor prognostic factor for the outcome of patients with low-risk intrathyroid papillary thyroid carcinoma: single-institution results from a large cohort study. J Clin Endocrinol Metab **97:**4390–4398.
16. Kebebew E, Weng J, Bauer J, Ranvier G, Clark OH, Duh QY, Shibru D, Bastian B, Griffin A 2007 The prevalence and prognostic value of *BRAF* mutation in thyroid cancer. Ann Surg **246:**466–470; discussion 470–461.
17. Kim TH, Park YJ, Lim JA, Ahn HY, Lee EK, Lee YJ, Kim KW, Hahn SK, Youn YK, Kim KH, Cho BY, Park do J 2012 The association of the *BRAF(V600E)* mutation with prognostic factors and poor clinical outcome in papillary thyroid cancer: a meta-analysis. Cancer **118:**1764–1773.
18. Prescott JD, Sadow PM, Hodin RA, Le LP, Gaz RD, Randolph GW, Stephen AE, Parangi S, Daniels GH, Lubitz CC 2012 *BRAF V600E* status adds incremental value to current risk classification systems in predicting papillary thyroid carcinoma recurrence. Surgery **152:**984–990.
19. Riesco-Eizaguirre G, Gutierrez-Martinez P, Garcia-Cabezas MA, Nistal M, Santisteban P 2006 The oncogene *BRAF V600E* is associated with a high risk of recurrence and less differentiated papillary thyroid carcinoma due to the impairment of Na+/I– targeting to the membrane. Endocr Relat Cancer **13:**257–269.
20. Xing M, Alzahrani AS, Carson KA, Viola D, Elisei R, Bendlova B, Yip L, Mian C, Vianello F, Tuttle RM, Robenshtok E, Fagin JA, Puxeddu E, Fugazzola L, Czarniecka A, Jarzab B, O'Neill CJ, Sywak MS, Lam AK, Riesco-Eizaguirre G, Santisteban P, Nakayama H, Tufano RP, Pai SI, Zeiger MA, Westra WH, Clark DP, Clifton-Bligh R, Sidransky D, Ladenson PW, Sykorova V 2013 Association between *BRAF V600E* mutation and mortality in patients with papillary thyroid cancer. JAMA **309:**1493–1501.
21. Yip L, Nikiforova MN, Carty SE, Yim JH, Stang MT, Tublin MJ, Lebeau SO, Hodak SP, Ogilvie JB, Nikiforov YE 2009 Optimizing surgical treatment of papillary thyroid carcinoma associated with *BRAF* mutation. Surgery **146:** 1215–1223.
22. Chen Y, Sadow PM, Suh H, Lee KE, Choi JY, Suh YJ, Wang TS, Lubitz CC 2016 *BRAF(V600E)* is correlated with recurrence of papillary thyroid microcarcinoma: a systematic review, multi-institutional primary data analysis, and meta-analysis. Thyroid **26:**248–255.
23. Lin KL, Wang OC, Zhang XH, Dai XX, Hu XQ, Qu JM 2010 The *BRAF* mutation is predictive of aggressive clinicopathological characteristics in papillary thyroid microcarcinoma. Ann Surg Oncol **17:**3294–3300.
24. Barbaro D, Incensati RM, Materazzi G, Boni G, Grosso M, Panicucci E, Lapi P, Pasquini C, Miccoli P 2014 The *BRAF V600E* mutation in papillary thyroid cancer with positive or suspected pre-surgical cytological finding is not associated with advanced stages or worse prognosis. Endocrine **45:** 462–468.
25. Dutenhefner SE, Marui S, Santos AB, de Lima EU, Inoue M, Neto JS, Shiang C, Fukushima JT, Cernea CR, Friguglietti CU 2013 *BRAF*: a tool in the decision to perform elective neck dissection? Thyroid **23:**1541–1546.
26. Jia Y, Yu Y, Li X, Wei S, Zheng X, Yang X, Zhao J, Xia T, Gao M 2014 Diagnostic value of *B-RAF(V600E)* in difficult-to-diagnose thyroid nodules using fine-needle aspiration: systematic review and meta-analysis. Diagn Cytopathol **42:**94–101.
27. Kim SK, Lee JH, Woo JW, Park I, Choe JH, Kim JH, Kim JS 2016 *BRAF V600E* mutation: differential impact on central lymph node metastasis by tumor size in papillary thyroid carcinoma. Head Neck **38:**E1203–1209.
28. Lubitz CC, Parangi S, Holm TM, Bernasconi MJ, Schalck AP, Suh H, Economopoulos KP, Gunda V, Donovan SE, Sadow PM, Wirth LJ, Sullivan RJ, Panka DJ 2016 Detection of circulating *BRAF(V600E)* in patients with papillary thyroid carcinoma. J Mol Diagn **18:**100–108.
29. Panka DJ, Buchbinder E, Giobbie-Hurder A, Schalck AP, Montaser-Kouhsari L, Sepehr A, Lawrence DP, McDermott DF, Cohen R, Carlson A, Wargo JA, Merritt R, Seery VJ, Hodi FS, Gunturi A, Fredrick D, Atkins MB, Iafrate AJ, Flaherty KT, Mier JW, Sullivan RJ 2014 Clinical utility of a blood-based *BRAF(V600E)* mutation assay in melanoma. Mol Cancer Ther **13:**3210–3218.
30. Rothenberg SM, McFadden DG, Palmer EL, Daniels GH, Wirth LJ 2015 Redifferentiation of iodine-refractory *BRAF V600E*-mutant metastatic papillary thyroid cancer with dabrafenib. Clin Cancer Res **21:**1028–1035.
31. Schlumberger M, Tahara M, Wirth LJ, Robinson B, Brose MS, Elisei R, Habra MA, Newbold K, Shah MH, Hoff AO, Gianoukakis AG, Kiyota N, Taylor MH, Kim SB, Krzyzanowska MK, Dutcus CE, de las Heras B, Zhu J, Sherman SI 2015 Lenvatinib versus placebo in radioiodine-refractory thyroid cancer. New Engl J Med **372:**621–630.
32. Shah JP, Clayman GL, Wirth LJ 2015 New and emerging therapeutic options for thyroid carcinoma. Clin Adv Hematol Oncol **13:**3–17, 11; quiz 12 p following 18.
33. Nucera C, Nehs MA, Mekel M, Zhang X, Hodin R, Lawler J, Nose V, Parangi S 2009 A novel orthotopic mouse model of human anaplastic thyroid carcinoma. Thyroid **19:**1077–1084.
34. Nucera C, Nehs MA, Nagarkatti SS, Sadow PM, Mekel M, Fischer AH, Lin PS, Bollag GE, Lawler J, Hodin RA, Parangi S 2011 Targeting *BRAFV600E* with PLX4720 displays potent antimigratory and anti-invasive activity in preclinical models of human thyroid cancer. Oncologist **16:** 296–309.
35. Routhier CA, Mochel MC, Lynch K, Dias-Santagata D, Louis DN, Hoang MP 2013 Comparison of 2 monoclonal antibodies for immunohistochemical detection of *BRAF V600E* mutation in malignant melanoma, pulmonary carcinoma, gastrointestinal carcinoma, thyroid carcinoma, and gliomas. Hum Pathol **44:**2563–2570.
36. Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD, Barletta JA, Wenig BM, Al Ghuzlan A, Kakudo K, Giordano TJ, Alves VA, Khanafshar E, Asa SL, El-Naggar AK, Gooding WE, Hodak SP, Lloyd RV, Maytal G, Mete O, Nikiforova MN, Nose V, Papotti M, Poller DN, Sadow PM, Tischler AS, Tuttle RM, Wall KB, LiVolsi VA, Randolph GW, Ghossein RA 2016 Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. JAMA Oncol **2:**1023–1029.
37. Xing M, Alzahrani AS, Carson KA, Shong YK, Kim TY, Viola D, Elisei R, Bendlova B, Yip L, Mian C, Vianello F, Tuttle RM, Robenshtok E, Fagin JA, Puxeddu E, Fugazzola L, Czarniecka A, Jarzab B, O'Neill CJ, Sywak MS, Lam

AK, Riesco-Eizaguirre G, Santisteban P, Nakayama H, Clifton-Bligh R, Tallini G, Holt EH, Sykorova V 2015 Association between *BRAF V600E* mutation and recurrence of papillary thyroid cancer. J Clin Oncol **33:**42–50.
38. Lupoli GA, Okosieme OE, Evans C, Clark PM, Pickett AJ, Premawardhana LD, Lupoli G, Lazarus JH 2015 Prognostic significance of thyroglobulin antibody epitopes in differentiated thyroid cancer. J Clin Endocrinol Metab **100:**100–108.
39. Juratli MA, Sarimollaoglu M, Nedosekin DA, Melerzanov AV, Zharov VP, Galanzha EI 2014 Dynamic fluctuation of circulating tumor cells during cancer progression. Cancers (Basel) **6:**128–142.
40. Sasportas LS, Hori SS, Pratx G, Gambhir SS 2014 Detection and quantitation of circulating tumor cell dynamics by bioluminescence imaging in an orthotopic mammary carcinoma model. PLoS One **9:**e105079.
41. Xu W, Wu B, Fu L, Chen J, Wang Z, Huang F, Chen J, Zhang M, Zhang Z, Lin J, Lan R, Chen R, Chen W, Chen L, Hong J, Zhang W, Ding Y, Okunieff P, Lin J, Zhang L 2017 Comparison of three different methods for the detection of circulating tumor cells in mice with lung metastasis. Oncol Rep **37:**3219–3226.
42. Ashida A, Sakaizawa K, Mikoshiba A, Uhara H, Okuyama R 2016 Quantitative analysis of the *BRAF V600E* mutation in circulating tumor-derived DNA in melanoma patients using competitive allele-specific TaqMan PCR. Int J Clin Oncol **21:**981–988.
43. Chang GA, Tadepalli JS, Shao Y, Zhang Y, Weiss S, Robinson E, Spittle C, Furtado M, Shelton DN, Karlin-Neumann G, Pavlick A, Osman I, Polsky D 2016 Sensitivity of plasma *BRAF*mutant and *NRAS*mutant cell-free DNA assays to detect metastatic melanoma in patients with low RECIST scores and non-RECIST disease progression. Mol Oncol **10:**157–165.
44. Denis MG, Knol A-C, Vallee A, Theoleyre S, Herbreteau G, Khammari A, Dréno B 2016 Cross-platform comparison of techniques to detect BRAF mutations in circulating tumor DNA of melanoma patients. J Clin Oncol **34:**e21026–e21026.
45. Kim BH, Kim IJ, Lee BJ, Lee JC, Kim IS, Kim SJ, Kim WJ, Jeon YK, Kim SS, Kim YK 2015 Detection of plasma *BRAF(V600E)* mutation is associated with lung metastasis in papillary thyroid carcinomas. Yonsei Med J **56:**634–640.
46. Reid AL, Freeman JB, Millward M, Ziman M, Gray ES 2015 Detection of *BRAF-V600E* and *V600K* in melanoma circulating tumour cells by droplet digital PCR. Clin Biochem **48:**999–1002.
47. Schreuer M, Meersseman G, Van Den Herrewegen S, Jansen Y, Chevolet I, Bott A, Wilgenhof S, Seremet T, Jacobs B, Buyl R, Maertens G, Neyns B 2016 Quantitative assessment of *BRAF V600* mutant circulating cell-free tumor DNA as a tool for therapeutic monitoring in metastatic melanoma patients treated with *BRAF*/*MEK* inhibitors. J Transl Med **14:**95.
48. Tsao SC, Weiss J, Hudson C, Christophi C, Cebon J, Behren A, Dobrovic A 2015 Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for *BRAF* and *NRAS* mutations. Sci Rep **5:**11198.
49. Brose MS, Cabanillas ME, Cohen EE, Wirth LJ, Riehl T, Yue H, Sherman SI, Sherman EJ 2016 Vemurafenib in patients with *BRAF(V600E)*-positive metastatic or unresectable papillary thyroid cancer refractory to radioactive iodine: a non-randomised, multicentre, open-label, Phase 2 trial. Lancet Oncol **17:**1272–1282.
50. Shah MH, Wei L, Wirth LJ, Daniels GA, Souza JA, Timmers CD 2017 Results of randomized Phase II trial of dabrafenib versus dabrafenib plus trametinib in *BRAF*-mutated papillary thyroid carcinoma. J Clin Oncol **35:**6022.
51. Subbiah V, Kreitman RJ, Wainberg ZA, Cho JY, Schellens JHM, Soria JC 2017 Efficacy of dabrafenib (D) and trametinib (T) in patients (pts) with *BRAF V600E*–mutated anaplastic thyroid cancer (ATC). J Clin Oncol **35:**6023.
52. Sherman EJ, Ho AL, Baxi SS, Dunn L, Korte SH, Haque S 2017 Combination of dabrafenib (DAB). J Clin Oncol **35:** 6085.

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