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Detection of Circulating *BRAF*^{V600E} in Patients with Papillary Thyroid Carcinoma

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BRAF^{V600E} is a common mutation in papillary thyroid carcinoma (PTC) correlated with aggressive features. Our objective was to assess the feasibility and accuracy of a novel RNA-based blood assay to identify individuals with a high-risk tumor mutation in patients with PTC. Patients with benign or malignant thyroid disorders were included between September 2013 and July 2014 before either thyroidectomy ($n = 62$) or treatment of recurrent or metastatic PTC ($n = 8$). RNA was isolated from peripheral blood lymphocytes and reverse transcribed and followed by two rounds of nested PCR amplification with a restriction digest specific for wild-type *BRAF*. *BRAF*^{V600E} levels were quantified with standardization curves. Circulating *BRAF*^{V600E} levels were compared with *BRAF* mutation status from surgical pathologic DNA-based tissue assays. Testing characteristics and receiving-operator curve using tissue results as the gold standard were assessed. Matched blood and tissue assays for *BRAF*^{V600E} were performed on 70 patients with PTC (stages I to IV, $n = 48$) or other ($n = 22$) thyroid tumors. Sixty-three percent of PTC patients tested positive for *BRAF*^{V600E} with conventional tissue assays on surgical specimens. The correlation between the RNA-based blood assay and tissue *BRAF* status was 0.71. PTC patients harbor detectable *BRAF*^{V600E} circulating tumor cells. This blood assay is feasible and has potential as a biomarker for prognosis, surveillance, clinical decision making, and assessment of treatment response to BRAF-targeted therapies. *(J Mol Diagn 2016, 18: 100—108; http://dx.doi.org/10.1016/j.jmoldx.2015.08.003)*

Thyroid cancer affects >530,000 individuals in the United States, and its incidence is increasing faster than any other cancer.[1] Thyroid cancer is the fifth most commonly diagnosed cancer in women (*http://www.cancer.org/research/cancerfactsfigures/cancerfactsfigures/cancer-facts-figures-2013*, last accessed July 14, 2015). Given that the number of deaths attributable to papillary thyroid cancer (PTC) is relatively stable, we are likely diagnosing a large number of patients that would otherwise not have become symptomatic or died as a result of their thyroid cancer.[2] Distinguishing those patients who may require more aggressive interventions from patients who may need less aggressive treatment would therefore be of great benefit.

Conventional thyroid cancer risk-stratification algorithms do not integrate mutational status as a predictor of risk.[3—5] *BRAF*^{V600E}, an activating mutation present in approximately one-half of PTCs, is highly specific for PTC among patients with thyroid nodules and is correlated with aggressive tumor features, recurrence of disease, loss of radioactive iodine (RAI) avidity, and increased mortality.[6—12] It remains controversial if prophylactic central cervical lymph node dissection improves outcomes in patients with PTC; however, given that

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$BRAF^{V600E+}$ patients were shown to have higher rates of central compartment metastases, some experts recommend the use of *BRAF* status to guide extent of initial surgery.[13–20] Moreover, the knowledge of *BRAF* status may be clinically actionable, because it can guide the extent of initial surgery (lobectomy versus total thyroidectomy and consideration of central lymphadenectomy), approach to imaging during surveillance (RAI scan versus positron emission tomography-computed tomography),[3] and adjuvant therapy. Furthermore, BRAF-targeted therapies for advanced thyroid cancers are being evaluated in clinical trials.[21–23]

Currently, fine-needle aspiration (FNA) or tissue biopsy is required for *BRAF* molecular testing and immunohistochemistry with anti-$BRAF^{V600E}$ antibodies (Abs).[24] Traditional tissue assays are considered less sensitive because of the potential for background tissue contamination. Compared with a routine blood draw, the FNA procedure, processing, and interpretation is costly and more challenging for patients. In addition, a blood-based assay would allow for easy access to serial, quantitative analysis to assess treatment effect and as a potential biomarker of recurrence. Our group has previously developed a highly sensitive blood-based $BRAF^{V600E}$ assay in patients with melanoma.[25]

Here, we report the feasibility of an RNA-based blood assay for the identification of individuals with a high-risk tumor mutation, $BRAF^{V600E}$, that previously could only be assessed invasively. We specifically hypothesize that an RNA-based blood $BRAF^{V600E}$ assay will be able to detect $BRAF^{V600E}$ from circulating tumor cells in patients with PTC. A sensitive blood-based $BRAF^{V600E}$ assay would provide an inexpensive and less-invasive mechanism for risk stratification, surveillance, and longitudinal assessment of treatment response. Ultimately, we believe that a rapid and easily ascertainable blood test for tumor $BRAF^{V600E}$ status may enable more targeted and resource-efficient management of patients with PTC.

Materials and Methods

Patient Selection

Under approval by the Partners Human Research Committee Institutional Review Board at the Massachusetts General Hospital, patients with benign ($n = 22$) and malignant ($n = 48$) thyroid disorders undergoing initial curative surgery or treatment of recurrent disease were enrolled between September 2014 and July 2014. After informed consent was obtained, a 5-mL sample of peripheral blood was obtained from each patient before surgery or before initiation with *BRAF*-inhibitor therapy for two patients with iodine-refractory metastatic disease and two patients after initiation of chemotherapy.

Protocol

The protocol in detail (reproduced from Panka et al[25] with permission from American Association for Cancer Research) is as follows.

Peripheral blood lymphocytes (PBLs) were isolated by Ficoll density centrifugation from each patient's pretreatment blood sample. These samples were stored in freezing medium (95% fetal calf serum with 5% DMSO) at −80°C. The $BRAF^{V600E}$ assay followed the protocol previously reported (Figure 1A).[25] Briefly, RNA from Ficoll purified PBLs was isolated by the Trizol method (Invitrogen, Grand Island, NY) and (3 μg) reverse transcribed to cDNA by standard methods using M-MLV reverse transcriptase (Invitrogen) and oligo (dt)15 (Promega, Madison, WI). The cDNA was subjected to real-time PCR for 18S RNA to normalize the quantity, as well as quality of the input RNA before the next step (ABI for oligo/probe set, Grand Island, NY). The equilibrated cDNA was PCR amplified using PCR master mix (Promega) and oligonucleotides [5′-CCATATCATTGAGACCAAATTTGAGATG-3′ (forward) and 5′-GGCACTCTGCCATTAATCTCTTCATGG-3′ (reverse)] that produced a product of 466 bp including the mutation site at position 600. The PCR conditions were 94° for 2 minutes followed by 40 cycles of 94° for 1 minute, 60° for 2 minutes and 72° for 2 minutes with a final incubation of 72° for 7 minutes. After clean up using a nucleospin extract column (Clontech, Mountain View, CA), a portion of the PCR product was digested with TSPR1 (restriction site = NNCASTGNN; New England Biolabs, Beverly, MA) at 65° for 16 hours. Only wild-type (WT) *BRAF* was digested by this enzyme. This digestion was added to reduce the amount of contaminating normal *BRAF* from surrounding and infiltrating normal tissue in the blood samples. A 1/100 dilution of the TSPR1 digested material was then PCR amplified a second time using nested oligonucleotides 5′-ACGCCAAGTCAATCATCCACAGAG-3′ and 5′-CCGTACCTTACTGAGATCTGGAGACAGG-3′ producing a product of 331 bp, which was enriched in PCR products containing the position 600 mutation. The conditions of the PCR were the same as the first PCR except the amplification was 45 cycles for PBLs instead of 40 cycles. After a second cleanup using a nucleo-spin extract column, the DNA (1/1000 dilution) was digested again with TspR1 and then subjected to a $BRAF^{V600E}$ real-time PCR as described. The annealing and extension temperature was adjusted to 64° resulting in a more favorable amplification of the mutant as compared to the WT templates than was reported. To further favor the mutant over the WT product, a 33-fold excess of the reverse (common sequence in mutant and WT) to forward (exact match for mutant and 1 base mismatch for WT sequences) primers were used in the real-time PCR assay.

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. Using the standard curve the amount of end product was determined. The RNA-based assay can reliably detect as low as 10 pg of $BRAF^{V600E}$ and has a 100-fold increased sensitivity compared to the WT PCR product (Figure 1B). Oligonucleotides were custom synthesized from Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO).

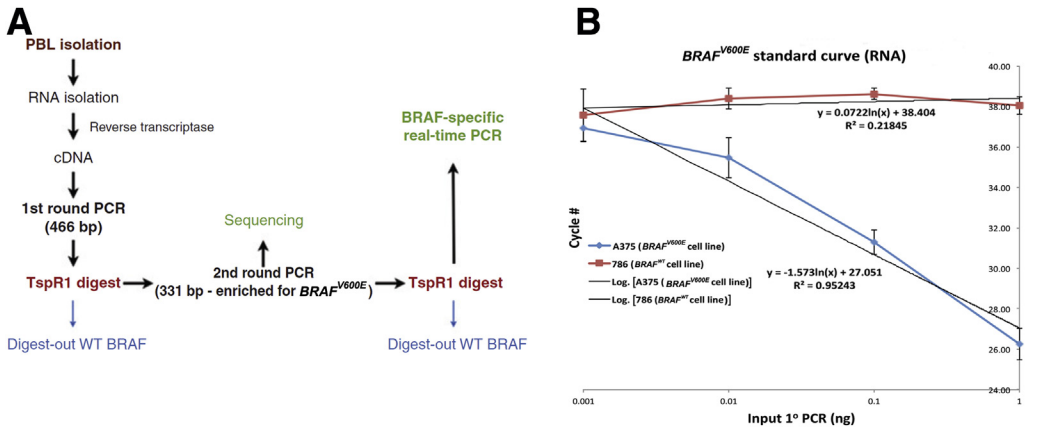

Figure 1 **A:** Schematic of the *BRAF*^{V600E} assay. **B:** Standard curve of the *BRAF* assay used for quantification. The equation representative of the best fit line for the *BRAF*^{V600E} (**lower equation**) and the wild-type (WT) *BRAF* (**upper equation**) are shown. Experiments were used to calculate the standard deviations of the a375 (*BRAF*^{V600E} hemizygous cell line) and 786 (*BRAF* wild-type cell line), respectively. $n = 5$ and 3 experiments. **Panel A** was reproduced from Panka et al[25] with permission from American Association for Cancer Research.

Assay development, testing, and validation were detailed previously.[26]

Tissue-Based BRAF Analysis

Patients with PTC had *BRAF* mutational analysis on tumor tissue as part of standard of care via SNaPshot (Massachusetts General Hospital Cancer Center Translational Research Laboratory).[27,28] For PTC patients in whom mutational status was not obtained at the discretion of the surgeon and for the benign lesions, *BRAF*^{V600E} mutation was sequenced from the 10-μm sections cut from formalin-fixed, paraffin-embedded tissue blocks. Tumor was verified in selected blocks by a pathologist (P.M.S.). The genomic DNA was isolated from the tissue sections with the use of QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This DNA is quantified and used in the PCR reaction with the use of primers forward, 5′-TCATAATGCTTGCTCTGATAGGA-3′, and reverse, 5′-GGCCAAAAATTTAATCAGTGGA-3′, to amplify a 200-bp product of the 15th exon region around the *BRAF*T1799A mutation with the use of high-fidelity Platinum PCR super mix (Invitrogen). The PCR product was later purified with QIAquick PCR purification kit (Qiagen) and sequenced with the forward PCR primer by the DNA core facility at Massachusetts General Hospital with the use of *ABI3730XL* DNA Analyzer. The data were visualized and analyzed with Finch TV software (Geospiza, Seattle, WA).

Thyroglobulin

Thyroglobulin levels were quantified for all patients by a commercial assay used in our institution (Mayo Medical Laboratories New England, Andover, MA).

Statistical Analysis

Clinical variables were chosen on the basis of established demographic and pathologic risk factors for decreased thyroid cancer-free survival. American Joint Committee on Cancer tumor, node, metastasis stage was calculated for each patient. For comparisons between *BRAF*^{V600E} and WT groups, pathologic 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 with Fisher's exact test, and continuous variables were assessed by Student's *t*-test or Wilcoxon rank sum test for nonparametric data. Comparisons of *BRAF* expression according to tissue mutational status and demographic and pathologic characteristics were based on linear regression models of natural log (*BRAF*). Tumor diameter, tumor volume, and age were considered as continuous variables. Statistical significance was defined as $P < 0.05$; no adjustments were made for multiple comparisons. With the use of tissue results as the gold standard, proportion of true positives (ie, sensitivity) and true negatives (ie, specificity) of the blood test in predicting the tissue results were calculated at various positivity thresholds. Likelihood ratios (sensitivity/1 − specificity) were calculated, and a receiver-operating curve was produced. An *a priori* decision was made to maximize specificity for determining the positivity criterion.

Results

Patient Characteristics

Seventy patients were enrolled in the study. Final surgical pathologic diagnoses were as follows: PTC ($n = 48$) (Table 1), follicular adenoma ($n = 9$), Hurthle cell neoplasm ($n = 4$), multinodular goiter ($n = 7$), minimally invasive follicular carcinoma ($n = 1$), and medullary thyroid carcinoma ($n = 1$). None of the patients had known melanoma or colorectal adenocarcinoma at enrollment. Patients with cancer had American Joint Committee on Cancer tumor, node, metastasis stages I to IV, including four patients with locally recurrent disease and four patients with iodine-refractory distant metastatic disease undergoing

Table 1 Clinical and Pathologic Characteristics of Patients

Characteristic	All PTC patients ($n = 48$)	PTC patients by $BRAF^{V600E}$ status ($n = 48$)		
		V600E ($n = 30$)	Wild-type ($n = 18$)	P value
Demographic characteristic				
Age, years, means ± SD	53 ± 16	55 ± 15	51 ± 18	0.47
Male, *n* (%)	15 (31)	13 (43)	2 (11)	0.03*
Primary tumor characteristics				
Tumor diameter, cm, median (IQR)	1.8 (1.3–2.5)	1.8 (1.4–2.6)	1.8 (1.1–3.1)	0.83
Multifocality, *n* (%)	28 (74)	21 (84)	7 (54)	0.06
Lymphovascular invasion, *n* (%)	23 (61)	21 (84)	2 (15)	<0.01*
Capsular invasion, *n* (%)	27 (71)	21 (84)	6 (46)	0.02*
Extrathyroidal extension, *n* (%)	11 (29)	8 (32)	3 (23)	0.71
Cervical lymph node status				
Central lymph node(s)$^+$, *n* (%)	16 (33)	13 (43)	3 (17)	0.07
Lateral lymph node(s)$^+$, *n* (%)	9 (19)	6 (20)	3 (17)	0.77
AJCC TNM stage				
I, *n* (%)	24 (50)	16 (53)	8 (44)	0.76
II, *n* (%)	4 (8)	2 (7)	2 (11)	
III, *n* (%)	12 (25)	8 (27)	4 (22)	
IV, *n* (%)	8 (17)	4 (13)	4 (22)	

*Statistically significant.
AJCC, American Joint Committee on Cancer; TNM, tumor, node, metastasis.

chemotherapy (with three of four patient with distant metastases undergoing treatment at the time of the blood draw, two $BRAF^{V600E+}$ patients with BRAF-inhibitor therapy, and one $BRAF^{WT}$ patient with recent treatment with a multitargeted tyrosine-kinase inhibitor). Compared with $BRAF^{WT}$ PTC patients, patients with $BRAF^{V600E}$ tumors on the basis of traditional tissue assays were more often men ($P = 0.03$) and had more lymphovascular ($P \leq 0.01$) and capsular ($P = 0.02$) invasion, indicating more aggressive tumors. Consistent with prior reports, a 63% prevalence of $BRAF^{V600E}$ mutation was found within the PTC patients with conventional tissue assays.

Two patients had false-positive tissue testing for $BRAF^{V600E}$ (with the use of surgical pathologic diagnosis as the gold standard). The indication for surgery in both of these patients was suspicious FNA biopsy (ie, suspicious for follicular neoplasm) with associate 30% risk of malignancy. Pathologic review (P.M.S.) of these two patients confirmed their diagnoses. The first patient, diagnosed as having a unilateral multinodular goiter with dominant adenomatous nodule (serum *BRAF* level, 7.2 pg), had only undergone thyroid lobectomy. The second patient (serum *BRAF* level, 7.2 pg) was subsequently diagnosed and treated for malignant melanoma.

Table 2 Correlation of Circulating ln($BRAF^{V600E}$) Levels and Other Covariates in PTC Patients ($n = 48$)

Variable	Coefficient	SE	Univariate R^2	P value
Demographic characteristics				
Age, years	0.041	0.024	0.061	0.096
Female	0.263	0.906	0.002	0.772
Primary tumor characteristics				
Tissue $BRAF^{V600E}$	2.260	0.801	0.148	<0.01*
Maximum tumor diameter, cm	−0.063	0.357	0.001	0.860
Tumor volume, cm^3	−0.059	0.104	0.010	0.567
Multifocality	0.456	0.968	0.006	0.640
Lymphovascular invasion	1.048	0.872	0.038	0.237
Capsular invasion	0.446	0.944	0.006	0.639
Extrathyroidal extension	−0.421	0.969	0.005	0.666
Cervical lymph node status				
Central lymph node(s)$^+$	0.703	0.885	0.014	<0.01*
Lateral lymph node(s)$^+$	0.744	1.071	0.010	0.491
AJCC-TNM stage	0.514	0.346	0.046	0.144

*Statistically significant.
AJCC, American Joint Committee on Cancer; TNM, tumor, node, metastasis.

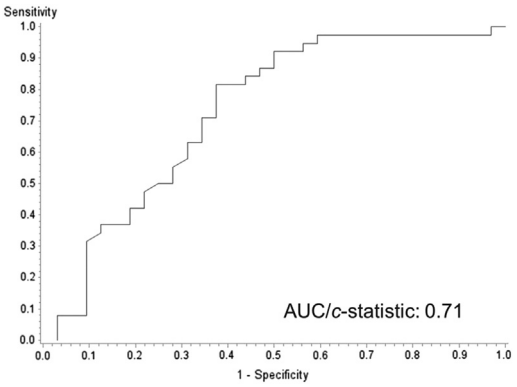

Figure 2 Receiver-operating analysis with each of the 70 circulating $BRAF^{V600E}$ levels as a possible positivity criterion in predicting the gold standard (tissue *BRAF* testing). AUC, area under the curve.

Correlation of Circulating $BRAF^{V600E}$ Levels with Patient and Tumor Characteristics

Linear regression analysis with demographic and pathologic predictors was performed in two subsets of patients: those in the cohort with PTC ($n = 48$) (Table 2) and those with PTC who were $BRAF^{V600E+}$ by conventional tissue assay ($n = 30$). Neither age nor sex was associated with blood $BRAF^{V600E}$ levels. Of the primary tumor characteristics, tissue *BRAF* positivity ($P = 0.007$) and central cervical lymph node positivity ($P = 0.003$) were correlated with circulating $BRAF^{V600E}$ levels in the first cohort (all PTC). Largest tumor diameter, tumor volume, and American Joint Committee on Cancer tumor, node, metastasis stage were not correlated with assay levels. Patients with benign disease had median serum *BRAF* levels 3.0 pg (interquartile range, 0.8 to 5.8 pg). The *BRAF* levels of patients who had a final pathologic diagnosis of PTC and were $BRAF^{V600E+}$ had a median blood $BRAF^{V600E}$ level of 17.3 pg (interquartile range, 1.9 to 127.5 pg), whereas in patients who had a PTC and were $BRAF^{-}$ the median blood $BRAF^{V600E}$ level was 2.0 pg (interquartile range, 0.5 to 4.4 pg).

Four patients in the study had distant metastatic disease, three of whom were $BRAF^{V600E+}$ by tissue testing. One patient with widely metastatic disease to the lung, liver, bone, and brain had a blood $BRAF^{V600E}$ level of 39,976 pg [thyroglobulin (Tg), 0.2 ng/mL]. This patient had been on treatment with BRAF-inhibitor therapy at the time of the blood draw for over a year, yet had progressive disease. The other two $BRAF^{V600E+}$ patients by tissue testing with distant metastatic disease had blood $BRAF^{V600E}$ levels of 8.2 pg (Tg, 68 ng/mL) and 442 pg (Tg Ab, >3000 IU/mL). Both of these samples were drawn before planned initiation with BRAF-inhibitor therapy. The last patient, with $BRAF^{WT}$ tumor (blood $BRAF^{V600E}$ level, 4.5 pg; Tg, 4193 ng/mL) had been treated with multitargeted receptor tyrosine kinase inhibitor, pazopanib, up until 3 months before the blood draw.

Testing Characteristics

With the use of the tissue *BRAF* result as the gold standard, likelihood ratios were calculated, and a receiver-operating characteristic curve for the blood assay predicting tissue *BRAF* mutational status was constructed (Figure 2 and Table 3). The area under the curve was 0.71, indicating moderate correlation of tests, assuming the tissue results are true. Correlation of tissue and blood assays with final surgical pathologic diagnosis is illustrated in Figure 3. As expected, the use of stricter (ie, higher) levels of circulating $BRAF^{V600E}$ decreased the sensitivity and increased the specificity. For example, the figure illustrates that most malignant PTC patients that were tissue typed as $BRAF^{V600E+}$ had circulating $BRAF^{V600E}$ levels in large excess of 10 pg. However, patients with benign tumor or patients with malignant tumors lacking the $BRAF^{V600E}$ mutation rarely had circulating $BRAF^{V600E}$ levels >10 pg.

Discussion

Clinical Implications and Utility of $BRAF^{V600E}$ Assay

Patients with PTC harboring the $BRAF^{V600E}$ mutation have more aggressive tumors and a reduced disease-free and overall survival.[6,7,9,13] Moreover, the $BRAF^{V600E}$ mutation is found in one-half of patients with PTC, making it a potentially useful biomarker in a large subgroup of thyroid cancer patients. Currently, *BRAF* status on the basis of the surgical pathology specimen is used by some clinicians to risk-stratify patients for recurrence.[13] Others have argued the use of *BRAF* mutational status from preoperative FNA specimens to guide extent of thyroidectomy (ie, lobectomy versus total thyroidectomy) and whether to perform prophylactic central lymphadenectomy.[14,15,29–32] Although a prospective trial to test the benefit of *BRAF* status to guide extent of surgery on outcome was not performed, we believe that *BRAF* status, in addition to other clinicopathologic risk factors, can be helpful in clinical decision making.

Traditional assays used to test *BRAF* require harvesting cytologic or pathologic tissue. The results of these tests are

Table 3 Sensitivity, Specificity, and Likelihood Ratio for Positive Tissue *BRAF* Test in Various Thresholds

$BRAF^{V600E}$ positivity threshold, pg	Sensitivity, %	Specificity, %	LR^{+}
5	62.5	71.1	2.2
10	50.0	86.8	3.9
20	43.8	92.1	5.5

With the use of tissue results as the gold standard, proportion of true-positive (ie, sensitivity) and true-negative (ie, specificity) results of the blood test in predicting the tissue results were calculated at various positivity thresholds. Likelihood ratios (sensitivity/1 − specificity) were calculated, and a receiver-operating curve was produced.

LR^{+}, likelihood ratio for positive tissue BRAF test.

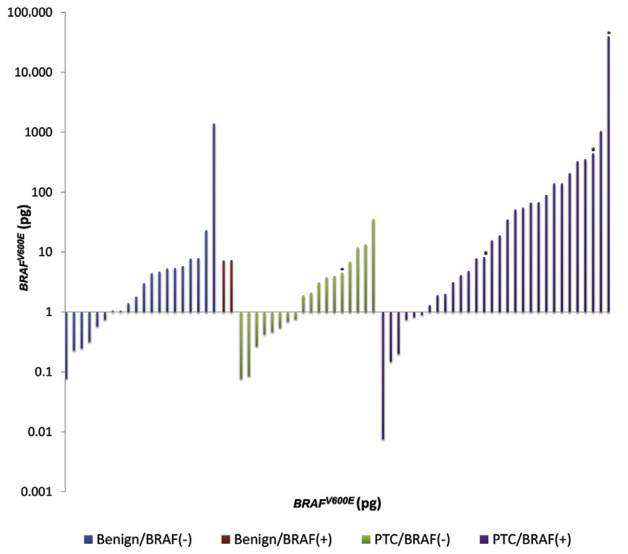

Figure 3 Circulating levels of $BRAF^{V600E}$ by pathologic diagnosis (benign, **left**; malignant, **right**) and tissue mutational status. Benign/$BRAF^{-}$ indicates a tumor that was read as either an adenoma or a benign hyperplastic lesion that is also negative for $BRAF^{V600E}$ by conventional tissue testing. Benign/$BRAF^{+}$ indicates pathologically negative, tissue $BRAF^{V600E+}$. **Asterisk** indicates patients with known distant metastatic disease.

limited by the lack of quantitative measurements and the potential for high background noise from surrounding stromal tissue.[33] In this study, we report the feasibility of an RNA-based assay for detection of $BRAF^{V600E}$ from circulating tumor cells. This assay has a number of advantages over current mutational testing and has potential clinical applications. In particular, blood-based biomarker detection has the advantage of allowing for serial measurements of circulating $BRAF^{V600E}$ that can be correlated with disease presence and/or severity over time. $BRAF^{+}$ tumors have more aggressive features and, in most series, have a higher incidence of disease recurrence.[7,8,13,34,35] Treatment of $BRAF^{+}$ recurrences are more difficult, however, because $BRAF^{+}$ tumors have impairment of the Na^{+}/I^{-} symporter necessary for RAI uptake. Increased uptake of RAI after *BRAFi* treatment in RAI-refractory patients was shown.[11,36,37] The ability to test circulating *BRAF* levels during treatment with *BRAFi* to assess response to treatment and to predict redifferentiation would be of great value in guiding continued treatment and choice of imaging. Finally, serial measurement of $BRAF^{V600E}$ may be particularly useful in patients with Tg-Abs (present in approximately 20% of PTC patients) in which Tg (conventional biomarker for PTC after thyroidectomy) levels are not useful as a biomarker.[38] Indeed, of the eight patients undergoing treatment for metastatic disease (ie, patients we had Tg levels available), one $BRAF^{+}$ patient undergoing treatment with *BRAFi* had elevated Tg Ab, making Tg levels useless as a biomarker. This same patient had a blood *BRAF* level of 442 pg, highlighting the application in this subset of patients. In addition, a small subset of patients with aggressive tumors ceases making Tg, rendering this assay potentially useful. This is illustrated by the one patient with known distant metastases who has a blood *BRAF* level of 39,976.0 pg but an almost undetectable Tg (0.2 ng/mL).

Improved Sensitivity with RNA-Based Assay

Our assay can detect mutant *BRAF* amid excess $BRAF^{WT}$. Previous blood-based assays for *BRAF* in melanoma patients were reported; however, they used DNA rather than RNA for the assay.[39–42] DNA is the preferred substrate for molecular testing in general because of its stability during handling in comparison with RNA. Vallachi et al[43] reviewed several $BRAF^{V600E}$ assays with the use of DNA as a substrate in metastatic melanomas. Most the assays were able to detect mutant *BRAF* at a frequency of 0.1% to 1% relative to $BRAF^{WT}$. Huang et al[44] examined formalin-fixed PTC tissue with the use of a DNA amplification refractory mutation system assay and were able to detect mutant *BRAF* at a frequency of 0.5% relative to $BRAF^{WT}$. Reported DNA-based assays for $BRAF^{V600E}$ in PTC patients have not been sensitive. Cradic et al,[45] only detected circulating $BRAF^{V600E}$ in 8 of 42 of the tumor-positive PTC patients during postoperative follow-up. Zane et al[46] did not identify any mutant *BRAF* in cell-free (ie, plasma-based assay) DNA in 46 patients with tissue-positive PTC. In addition to having exponentially higher copy numbers of *BRAF* mRNA, digestion of $BRAF^{WT}$ with TspRI in our assay further increased the sensitivity for detection. We have performed a direct comparison of usage of RNA with DNA as substrates in this assay. On the basis of the data from the standard curves $BRAF^{WT}$ is not detected in this assay with the use of up to 1 ng of DNA from the first round of PCR. The best fit lines of the $BRAF^{V600E}$ and $BRAF^{WT}$ intersect at 1 pg but are statistically significantly different at 10 pg ($P < 0.03$), which represents the lower limit of detection of the $BRAF^{V600E}$. We therefore concluded that the $BRAF^{V600E}$ can be statistically detected at a frequency of 10 pg V600E in 1000 pg of $BRAF^{WT}$ or 1%. By comparison, following the same procedure except for the oligonucleotides for the first two PCRs and with the use of genomic DNA as a substrate and a statistically significant lower limit of 10 pg, the assay could only detect the mutant at a frequency of 3% relative to the $BRAF^{WT}$, a difference that in some patient samples prevented the detection of the mutant *BRAF* in the blood (D.J.P., personal communication; data not shown). The use of RNA and digestion of $BRAF^{WT}$ enhances the sensitivity of our assay.

Importantly, we have shown that circulating $BRAF^{V600E}$ is detectable even in patients with minimal tumor burden. Even in the 10 patients without extrathyroidal extension or lymph node metastases and tumors <2 cm (T1 tumors) that were $BRAF^{+}$ on tissue testing, 6 had blood *BRAF* levels >10 pg (all were detectable). In comparison with the population of patients with PTC in the United States, our cohort had more patients with stage III and IV tumors. However, reflective of national trends, most patients in this study had small tumors (<2.0 cm) and were female. We found a

relatively high prevalence of $BRAF^{V600E}$ mutation in our group, perhaps accounted for by the over-representation of patients with more aggressive tumors. This mutation is common and highly specific for PTC among patients with thyroid nodules. Tissue $BRAF^{V600E}$ status in our study was correlated with male patients, lymphovascular invasion, and capsular invasion. When correlating the blood *BRAF* levels with the same clinical and pathologic variables, these three variables were not correlated. As expected, good correlation was found between tissue *BRAF* status and level of circulating $BRAF^{V600E}$.

Although the assay appears feasible, findings warrant discussion. Of concern were the two patients that had a final pathologic diagnosis of multinodular goiter with $BRAF^{V600E+}$ tissue testing (blood *BRAF* levels were 7.2 and 7.4 pg). Both patients had surgery for FNA biopsies that were suspicious for follicular neoplasm which portends a 30% risk of malignancy. On review of the surgical pathology for these two patients it was noted that one patient had a papillary microcarcinoma (1 mm in diameter) that may have been the source of $BRAF^{V600E}$ in both the tissue and the blood. $BRAF^{V600E}$ is detectable even in papillary thyroid microcarcinomas (PTC $<$ 1 cm).[47–49] No further intervention is required for this patient. The situation for the second patient is less clear. Although the surgical pathology does not reveal any PTC (unless missed by the pathologist), the patient was subsequently diagnosed and treated for malignant melanoma, potentially explaining the elevated blood level but not the thyroid tissue result. In addition, this patient had only a thyroid lobectomy. The circulating $BRAF^{V600E}$ could potentially be caused by an occult PTC in the contralateral lobe. As with our prior work in a group of patients with malignant melanoma, wherein mean blood $BRAF^{V600E}$ levels were detectable but low (ie, $BRAF^{WT}$, 1.7 pg; controls, 1.2 pg; $BRAF^{V600E}$, 50.3 pg), we found detectable circulating $BRAF^{V600E}$ in patients with benign disease.[25] Finally, one patient had a thyroid lobectomy for a Hurthle cell neoplasm and a $BRAF^{V600E}$ level of 1384 pg; a possibility of a $BRAF^{V600E+}$ tumor in the contralateral unresected lobe or occult melanoma, benign nevi, or serrated colorectal adenoma or colorectal carcinoma, all known to have $BRAF^{V600E}$ mutations, cannot be eliminated.[25,50–53] Outliers may be considered for evaluation for occult cancers.

Determining the Optimal Positivity Criterion

The receiver-operating characteristic analysis and area under the curve (c-statistic $= 0.71$) indicates a moderate ability of our RNA-based blood assay to predict the *BRAF* status as assessed by DNA-based conventional tissue assays. As shown in Figures 2 and 3, increasing thresholds for a positive blood test increases the specificity (ie, low false-positive rate). However, prevalence of disease (in this case prevalence of $BRAF^{V600E}$ in the PTC population) and the consequences of false-positive and false-negative results should be considered when choosing a positivity criterion. Given the minimal impact on clinical care with a false-negative result and that a false-positive result could result in potential unwarranted treatment, we would choose to optimize specificity (ie, a higher threshold). Unlike premalignant nevi, in which $BRAF^{V600E}$ has been detected, $BRAF^{V600E}$ is thought to be highly specific for PTC in thyroid nodules.[43]

Study Limitations

In assessing the accuracy of the blood assay, we used tissue *BRAF* status as the gold standard, making the assumption that this test is perfect. Although we are left without an alternative gold standard by which to assess the accuracy of the blood assay, it is possible that the blood assay is more sensitive and specific than the tissue samples, given the potential for high background from sampling of stromal areas of the tumor normally surrounding thyroid in the tissue specimens. In addition, we cannot entirely exclude the possibility that detectable circulating levels of $BRAF^{V600E}$ are from an undiagnosed dermal or colorectal disorder. None of the patients in our study had known concomitant cancers at the time of enrollment, and one patient subsequently was diagnosed with a malignant melanoma (within months of thyroidectomy).

Conclusions

We have shown that an RNA-based $BRAF^{V600E}$ is feasibly detectable in the blood patients with PTC, even with low tumor burden. This assay has a number of potential clinical applications. Given that $BRAF^{V600E}$ has not been found in benign thyroid tissue or adenomas, high blood $BRAF^{V600E}$ levels could be used as a preoperative adjunct to FNA to guide initial extent of surgery. A highly specific, less costly blood test could be an alternative to other costly, send-out molecular diagnostics. Thyroid cancer is a common disease with a clinical need for improved risk stratification for most patients who do well. Next, by quantifying the change in levels after surgery for $BRAF^{+}$ patients clinicians could assess completeness of surgery, residual disease in Tg Ab^{+} patients, need for or utility of adjuvant RAI, or response to adjuvant BRAF-targeted therapy. Moreover, in the small but substantial group of patients destined to do poorly, there is an enrichment of *BRAF* mutations. Thus, the ability to accurately detect and follow *BRAF* levels over time as a biomarker for recurrence and response to *BRAF* inhibitors would be of great clinical value. Specifically, we previously reported the accuracy of this assay in a cohort of patients with melanoma wherein we were able to show that patient *BRAF* levels declined after treatment with BRAF-inhibitor therapy and that serial measurements allowed biochemical detection before structural disease.[25] Cradic et al[45] found a correlation with detectable *BRAF* in patients with known

residual PTC. Moreover, should BRAF-targeted therapy prove beneficial in patients with *BRAF*-mutant advanced thyroid cancer, the assay may be useful as an early predictor of response to therapy.

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