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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).