Supplemental Online Content

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This supplemental material has been provided by the authors to give readers additional information about their work.
eMethods.

1. Thyroid Cancer Cells Harboring Wild-type and Variant BRAF

Thyroid cancer cell lines FTC-133 and BCPAP\(^1\), respectively harboring wild-type BRAF and variant BRAF V600E (kindly provided by Dr. Shilpa Thakur, NIH, MD, US), were used for extraction of genomic DNA as the negative and positive controls for verifying the detection of BRAF variations. FTC-133 cells were cultured in DMEM growth medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and BCPAP cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with 5% FBS, each containing 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified 5% CO\(_2\) incubator. The cell authentication of two cell lines originated from human thyroid follicular (FTC-133) and papillary cancers (BCPAP) were confirmed by short tandem repeat profiling analysis by TCAG Genetic Analysis Facility (The Centre for Applied Genomics, Toronto, CAN).

2. Genomic DNA Extraction from Thyroid Cancer Cells, FFPE and FNA Biopsies

Monolayer FTC-133 or BCPAP cell culture were detached at 90% confluence, then fixed in CytoLyte solution overnight at room temperature to mimic the procedure of preparation of FNA biopsies. FTC-133 or BCPAP cells and the remaining material of FNA biopsies preserved in CytoLyte solution were pelleted first and re-suspended in 200 µl of PBS for DNA isolation using the DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany), according to manufacturer's instructions. FFPE tissue blocks were sectioned at 10-µm thickness, and DNA extraction was performed with QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). All patient samples were de-identified. The DNA isolation and subsequent testing were all blinded to the patients’
demographics, diagnosis, and disease status. The extracted DNA elute was stored at -20°C. DNA concentration was measured using NanoDrop 8000 (Thermo Fisher Scientific).

### 3 Immunohistochemistry for the BRAF V600E Variation

Immunohistochemistry (IHC) staining was performed using FFPE tissue sections (4μm) and the anti-BRAF\textsuperscript{V600E} (VE1) antibodies (Roche Diagnostics, ON, CAN) on the Ventana Benchmark Ultra (Ventana Medical Systems, AZ, USA) with Cell Conditioning 1 for 64 min, pre-peroxidase inhibition and primary antibody incubation for 16 min at 37°C according to the manufacturer’s procedures in Mount Sinai Services (MSH, ON, CAN). The OptiView Universal DAB IHC Detection Kit (Ventana Medical Systems) was used to detect BRAF V600E expression on the Ventana Benchmark Ultra autoimmunostainer\textsuperscript{3}. Tissues were manually counterstained with CAT Hematoxylin and Bluing Reagent (Biocare Medical, CA, USA) for 2 min. PTC tissues harboring the BRAF V600E variation and tissues lacking BRAF V600E variation identified by direct Sanger sequencing, were included in the staining procedure in every batch. Stained slides were scanned by Microscopy Slide Scanner ZEISS Axio Scan.Z1 (Zeiss, Oberkochen, Germany) in the OPTical IMAging (OPTIMA) Facility at Lunenfeld-Tanenbaum Research Institute in Mount Sinai Hospital and all H&E or immunostaining images were acquired via ZEISS ZEN (blue edition). Slides were evaluated by an experienced pathologist who was blinded to the BRAF mutational status. BRAF V600E immunostaining was scored by combining percentage scale and overall intensity level of cells staining positive\textsuperscript{4}. The percentage scale was determined by the percentage of cells staining positive in cytoplasm according to: 0 (≤ 10%), 1 (11–30%), 2 (31–50%), 3 (51–70%), or 4 (> 70%), and the intensity level of stained cells was graded as 1= weak or focal cytoplasmic staining, 2 = moderate or 3 = strong. The total score value ranges from 0 to
7. Equivocal or no staining in tumor cells and non-specific staining of colloids were considered negative.

4. **Assessment of Sensitivity, Specificity, and Positive and Negative Predictive Values**

The accuracy and reproducibility of dPCR detection of BRAF V600E was further assessed using serial dilutions of DNA samples from FTC-133 cells mixed with 1%, 5% and 10% of DNA from BCPAP cells, respectively, and serial dilutions of DNA samples from clinical specimens. BRAF VAF was calculated by BRAF variant alleles (copies) vs. the total copies of BRAF variant alleles and BRAF wild-type alleles in each reaction from all triplicate wells. The analytical specificity was validated with a temperature gradient to ensure 6-FAM LNA probe to specifically detect BRAF variant alleles and HEX LNA probe specifically detecting BRAF wild-type alleles, as well as each probe showing no cross-reactivity between variant and wild-type clusters using FTC-133 and BCPAP DNA by 2-dimensional plot analysis. The optimal annealing temperature is determined by the condition in which the variant 6-FAM probe exhibits no false positives in the wild-type FTC-133 DNA samples and relative distance between the 6-FAM-only and HEX-only clusters is maximal. The specificity was further confirmed by Sanger sequencing of PCR amplified products (Eurofins Genomics, KY, USA). To assess the detection sensitivity, dPCR reactions were performed with 50 ng of input DNA at serial dilutions (ie. 0, 0.3%, 1%, 3%, 10%, 30%, and 100%) of DNA from BRAF variant BCPAP cells in the background of BRAF wild-type DNA from FTC-133 cells. For example, the 0.3% mix contained 0.3% of BCPAP DNA and 99.7% of FTC-133 DNA. Sensitivity was determined by the limit of detection (LoD), the analytical lowest level, at which the variant allele DNA could be reliably distinguished from a blank (zero concentration) sample (LoB). A serial of varying concentrations of BRAF variant
alleles (copies/ul) vs. serial dilutions of input variant DNA (%) was achieved to determine the model of a linear calibration curve as well as the LoB and LoD. The LoB was estimated using the following formula: LoB = Mean\text{blank} + 1.645\times (SD\text{blank}). The Mean\text{blank} and SD\text{blank} are calculated using replicates, up to 20 wells of a zero calibrator or blank sample (0% variant DNA and 100% wild-type DNA). The LoD was calculated by analyzing triplicates from three separate experiments and using the following formula: LoD = LoB + 1.645\times (SD\text{0.3\% sample}). The reproducibility was expressed as coefficient of variance (CV) of standard deviation of BRAF V600E detection result. The CV was quantified using the following formula: CV = SD/Mean. The clinical sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals of the residual FNA-assessed BRAF variation status for tumor malignancy were calculated using standard formulas in accordance with STARD 2015.

Availability of Methods and Materials

Further information and requests for dPCR assays should be directed to and will be fulfilled by the Lead Contact, Guodong Fu (David.Fu@sinaihealth.ca; gdfu2002@gmail.com).

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**eTable 1. Demographic and Clinicopathological Features of Patients and BRAF V600E in Thyroid PTC by Droplet dPCR Assay and IHC Staining**

| Characteristics | Patient, No. (%) |  |  |  |  |  |  |  |  |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Total (n = 77)  | Benign (n = 4)  | NIFTP (n = 23)  | FVPTC (n = 36)  | cPTC (n = 14)  |  |  |  |  |
| **Sex**         |  |  |  |  |  |  |  |  |  |
| Women           |  |  |  |  |  |  |  |  |  |
| Women           |  |  |  |  |  |  |  |  |  |
| Men             |  |  |  |  |  |  |  |  |  |
| Men             |  |  |  |  |  |  |  |  |  |
| **Age, Mean (SD), y** |  |  |  |  |  |  |  |  |  |
| < 45            | 59 (76.6)      | 2 (3.4)         | 17 (28.8)       | 29 (49.2)       | 11 (18.6)      | .547            |  |  |  |
| ≥ 45            | 18 (23.4)      | 2 (11.1)        | 6 (33.3)        | 7 (38.9)        | 3 (16.7)       |                |  |  |  |
| **Age category, y** |  |  |  |  |  |  |  |  |  |
| < 45            | 35 (45.5)      | 2 (5.7)         | 9 (25.7)        | 16 (45.7)       | 8 (22.9)       | .775            |  |  |  |
| ≥ 45            | 42 (54.5)      | 2 (4.8)         | 14 (33.3)       | 20 (47.6)       | 6 (14.3)       |                |  |  |  |
| **Thyroidectomy** |  |  |  |  |  |  |  |  |  |
| Total           | 66 (88.0)      | 2 (3.0)         | 19 (28.8)       | 34 (51.5)       | 11 (16.7)      | .003            |  |  |  |
| Partial         | 9 (22.0)       | 2 (22.2)        | 4 (44.4)        | 0               | 3 (33.3)       |                |  |  |  |
| **Tumor size, Mean (SD), cm** |  |  |  |  |  |  |  |  |  |
| < 45            | 2.86 (1.71)    | 2.60 (2.99)     | 2.97 (1.94)     | 2.76 (1.42)     | 3.02 (1.78)    | .935            |  |  |  |
| ≥ 45            | 2.86 (1.71)    | 2.60 (2.99)     | 2.97 (1.94)     | 2.76 (1.42)     | 3.02 (1.78)    |                |  |  |  |
| **Encapsulated** |  |  |  |  |  |  |  |  |  |
| No              | 7 (9.3)        | 2 (28.6)        | 0               | 0               | 5 (71.4)       | <.001           |  |  |  |
| Yes             | 68 (90.7)      | 0               | 23 (33.8)       | 36 (52.9)       | 9 (13.2)       |                |  |  |  |
| **BRAF V600E IHC** |  |  |  |  |  |  |  |  |  |
| Score = 0       | 64 (83.1)      | 4 (6.2)         | 23 (35.9)       | 29 (45.3)       | 8 (12.5)       | <.001           |  |  |  |
| Score < 3       | 3 (3.9)        | 0               | 0               | 3 (100)         | 0 (0)          |                |  |  |  |
| Score ≥ 3       | 10 (13.0)      | 0               | 0               | 4 (40.0)        | 6 (60.0)       |                |  |  |  |
| **BRAF V600E dPCR** |  |  |  |  |  |  |  |  |  |
| 0 VAF           | 61 (79.2)      | 4 (6.6)         | 23 (37.7)       | 26 (42.6)       | 8 (13.1)       | .005            |  |  |  |
| < 1% VAF        | 4 (5.2)        | 0               | 0               | 4 (100)         | 0 (0)          |                |  |  |  |
| ≥ 1% VAF        | 12 (15.6)      | 0               | 0               | 6 (50.0)        | 6 (50.0)       |                |  |  |  |

Abbreviations: cPTC: classical variant papillary thyroid cancer; FVPTC: follicular variant papillary thyroid cancer; NIFTP: non-invasive follicular thyroid neoplasm with papillary-like nuclear features; VAF: Variant allele fraction.

*a* Missing data for 2 patients owing to incomplete information.

*b* Fisher's exact test (2-sided) for categorial variables and ANOVA test for independent parametric continuous measures.
eTable 2. BRAF V600E Variation Status in Indeterminate FNAs with Demographics and Clinicopathological Features

| Characteristics            | Patient, No. (%) |   |   |   | P value* |
|----------------------------|------------------|---|---|---|----------|
|                            | Total (n = 146)  | ND (n = 50) | AUS/FLUS (n = 58) | SFM (n = 11) | Malignant (n = 27) |
| Sex                        |                  |              |                  |             |          |
| Women                      | 115 (78.8)       | 40 (34.8)    | 48 (41.7)        | 10 (8.7)    | 17 (14.8) |
| Men                        | 31 (21.2)        | 10 (32.3)    | 10 (32.3)        | 1 (3.2)     | 10 (32.3) |
| Age Mean (SD), y           | 55.7 (15.8)      | 58.7 (15.4)  | 54.9 (16.1)      | 54.7 (15.2) | 52.0 (15.8) |
| Age category, y            |                  |              |                  |             |          |
| < 45                       | 31 (21.2)        | 7 (22.6)     | 13 (41.9)        | 2 (6.5)     | 9 (29.0)  |
| ≥ 45                       | 115 (78.8)       | 43 (37.4)    | 45 (39.1)        | 9 (7.8)     | 18 (15.7) |
| Source of biopsies         |                  |              |                  |             |          |
| Left side                  | 60 (41.1)        | 21 (35.0)    | 24 (40.0)        | 3 (5.0)     | 12 (20.0) |
| Right side                 | 81 (55.5)        | 27 (33.3)    | 31 (38.3)        | 8 (9.9)     | 15 (18.5) |
| Isthmus                    | 5 (3.4)          | 2 (40.0)     | 3 (60.0)         | 0           | 0         |
| DNA yield Mean (SD), µg    | 0.87 (1.08)      | 0.81 (1.26)  | 0.97 (1.09)      | 0.91 (0.77) | 0.74 (0.75) |
| BRAF VAF category          |                  |              |                  |             |          |
| 0 VAF                      | 107 (73.3)       | 41 (38.3)    | 44 (41.1)        | 7 (6.5)     | 15 (14.0) |
| < 1% VAF                   | 21 (14.4)        | 4 (19.0)     | 11 (52.4)        | 3 (14.3)    | 3 (14.3)  |
| ≥ 1% VAF                   | 18 (12.3)        | 5 (27.8)     | 3 (16.7)         | 1 (5.6)     | 9 (50.0)  |

Abbreviations: ND, Insufficient for diagnosis; AUS/FLUS, Atypia of undetermined significance/Follicular atypia of undetermined significance; SFM, Suspicious for malignancy; VAF, Variant allele fraction.

*2-sided Fisher exact test for categorial variables and ANOVA test for parametric continuous measures.
eFigure 1. Optimization of Droplet dPCR Conditions

A

B

69.0°C

66.7°C

63.5°C

59.6°C

56.4°C

54.2°C

53.0°C

69.0°C

66.7°C

63.5°C

59.6°C

56.4°C

54.2°C

53.0°C

69.0°C

66.7°C

63.5°C

59.6°C

56.4°C

54.2°C

53.0°C

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1-dimensional (A) and two-dimensional plots (B) of droplet dPCR were shown using BRAF98/LNA probes at gradient temperatures (53°C-69°C) with 50ng of BCPAP DNA alone (left column), 50ng of FTC-133 DNA (middle column) or mix of 50ng of BCPAP and FTC-133 DNA each (right column) in A&B panels. Red circles marked nonspecific positive droplets at 53°C-56.4°C in panel B. In 2-Dimensional plot, droplets were divided into distinct clusters separated by blue dots for 6-FAM positive droplets, green dots for HEX positive droplets and grey dots for negative droplets with thresholds across both channel 1 at 2500 and channel 2 at 2000.
**eFigure 2.** Droplet dPCR Based on LNA Probes Failed in Specifically Detecting BRAF V600E Variation Using Primer Set BRAF91

[A and B. 1-dimensional plot showed droplet dPCR amplification of genomic DNA of BCPAP cell alone (A) or genomic DNA mix (at ratio 1:1) from BCPAP and FTC-133 cells (B) at gradient annealing temperatures from 53°C to 69°C using primer set BRAF91/LNA probes. Blue dots indicated signal from the specific binding of the LNA FAM variant probes to BRAF V600E (in upper panels of A and B) and green dots indicated signal from the specific binding of LNA HEX wild-type probes to wild-type BRAF (in lower panels of A&B), while negative droplets are displayed in grey dots. C and D, 2-dimensional plot of droplet dPCR using primer set BRAF91/LNA probes at 59.6°C with 50ng of BCPAP DNA alone (C) or mix of 50ng of BCPAP and FTC-133 DNA each (D). Non-specific amplified droplets were marked in red circle. In 2-Dimensional plot, droplets were divided into distinct clusters separated by blue dots for 6-FAM positive droplets, green dots for HEX positive droplets and grey dots for negative droplets with thresholds across both channel 1 at 2500 and channel 2 at 2000.]
**eFigure 3.** BRAF V600E Variation in Surgical FFPE Tissue Was Concordantly Detected by Both IHC Staining and dPCR Assay

IHC and dPCR analyses of BRAF V600E variation in subtypes of PTC, benign and NIFTP tissue with a correlation between BRAF VAF and BRAF V600E staining. Top panel (H&E) demonstrated morphological features of examined tumors, and middle and bottom panels showed results of BRAF IHC staining and dPCR assay, respectively. The *BRAF* VAF quantified by dPCR assays was 0 in benign, 0 in NIFTP, 25.1% in EFVPTC, 13.3% in cPTC and 37.9% cPTC with EFG. In 2-Dimensional plot, droplets were divided into distinct clusters separated by blue dots for 6-FAM positive droplets, green dots for HEX positive droplets and grey dots for negative droplets with thresholds across both channel 1 at 2500 and channel 2 at 2000.

Abbreviations: VAF, Variant allele fraction; NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; EFVPTC, encapsulated follicular variant papillary thyroid cancer; cPTC, classic variant papillary thyroid cancer; and EFG, extensive follicular growth.
**eFigure 4.** Scatter Diagram of IHC and dPCR with Regression Line

\[ y = 0.1728x + 0.2303 \]
\[ R^2 = 0.7882 \]

*BRAF* variant allele fraction (VAF) (%) Line Fit Plot showed the *BRAF* variation status:
Predicted IHC score = 0.173 *BRAF* VAF (%) + 0.230; \( R^2 = 0.79; p < .0001 \), with a Pearson Correlation = 0.90, \( p < .001 \).
eFigure 5. 2-Dimensional Plot of BRAF V600E Variation in the Residuals of FNA Samples and Matched Surgical Specimens by dPCR
The left panel of A-F demonstrated dPCR detection of BRAF V600E variation in the residual tissue of FNA samples and quantification of BRAF VAF at 0 in benign FNA residual tissue (A), 0 in ND FNA residual tissue (B), 0.14% in AUS FNA residual tissue (C), 0.13% in FLUS FNA residual tissue (D), 25.9% in SFM FNA residual tissue (E) and 18.6% in malignant FNA residual tissue (F). The right panel of G-L demonstrated dPCR detection of BRAF V600E variation in the matched surgical specimens and quantification of BRAF VAF at 0 in benign matched surgical specimens (G), 0 in ND matched surgical specimens (H), 7.92% in AUS matched surgical specimens (I), 0.20% in FLUS matched surgical specimens (J), 22.8% in SFM matched surgical specimens (K) and 24.2% in malignant matched surgical specimens (L). The residual of benign FNA samples (A) and its matched surgical specimens (G) were included as control samples in the test. In 2-Dimensional plot, droplets were divided into distinct clusters separated by blue dots for 6-FAM positive droplets, green dots for HEX positive droplets and grey dots for negative droplets with thresholds across both channel 1 at 2500 and channel 2 at 2000. Abbreviations: VAF, Variant allele fraction; ND, Insufficient for diagnosis; AUS/FLUS, Atypia of undetermined significance/ Follicular atypia of undetermined significance; SFM, Suspicious for malignancy.