

# Circulating *BRAF* V600E Cell-Free DNA as a Biomarker in the Management of Anaplastic Thyroid Carcinoma

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**abstract** **Purpose** Anaplastic thyroid cancer (ATC) is a deadly form of thyroid cancer. *BRAF* V600E is the only actionable mutation for which there is a Food and Drug Administration–approved drug combination. Rapid detection of *BRAF* V600E and initiation of therapy is critical. We explored the ability of droplet digital polymerase chain reaction (ddPCR) to identify this mutation in circulating cell-free DNA (cfDNA) in plasma.

**Materials and Methods** The ddPCR assay was evaluated for its sensitivity, specificity for detection of *BRAF* V600E cfDNA, and concordance with tumor tissue. The assay also was used to evaluate its potential role as a biomarker of response.

**Results** Forty-four patients with ATC who were tested for the *BRAF* mutation by tumor tissue DNA sequencing or immunohistochemistry were included. Sixteen *BRAF* V600E–positive patients had treatment samples to evaluate cfDNA levels as a biomarker of response in correlation with restaging scans. Concordance of ddPCR with tumor tissue was 93%, with a sensitivity of 85% and specificity of 100%. Area under the curve by Wilcoxon rank sum test was 0.9 (95% CI, 0.80 to 0.99;  $P < .001$ ). As a biomarker of response to treatment, 94% of ddPCR samples were concordant with tumor shrinkage in restaging scans, and 47% were concordant with tumor growth (Fisher’s exact test  $P = .0061$ ). In addition, cfDNA levels by ddPCR were predictive of treatment response in 71% of samples.

**Conclusion** cfDNA detection by ddPCR is highly sensitive, specific, and concordant with mutation status on ATC tumors. ddPCR also can be used for monitoring cfDNA levels in conjunction with imaging scans in patients with ATC.

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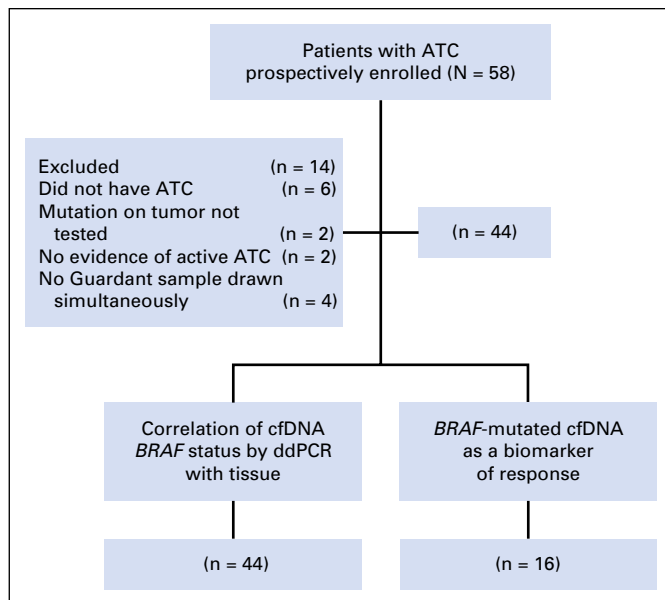
## INTRODUCTION

Anaplastic thyroid carcinoma (ATC) is responsible for one third of thyroid cancer deaths and has a high mortality rate.<sup>1,2</sup> *BRAF* V600E is the most common actionable driver mutation in ATC, which is seen in approximately 45% of these cancers.<sup>3,4</sup> Indeed, targeted treatment strategies are extending patient survival,<sup>5–8</sup> and the *BRAF* plus MEK inhibitor combination dabrafenib plus trametinib was recently approved by the Food and Drug Administration for *BRAF* V600E–mutated ATC.<sup>9,10</sup>

Diagnosis of ATC usually is made by biopsy. Unlike differentiated tumors, the lack of sufficient viable tissue hampers molecular diagnostic

testing.<sup>2,11,12</sup> Given the rapid progression of ATC, rapid testing is needed for timely therapeutic intervention. Liquid biopsy (mutation testing performed on blood samples) provides a potential alternative to tissue biopsy in the management of certain malignancies.<sup>13–15</sup> The detection of mutations in circulating cell-free DNA (cfDNA), such as *BRAF* V600E, can serve as a surrogate for mutation testing on tissue biopsy, and its overall levels provide a novel marker in predicting thyroid cancer burden.<sup>16,17</sup>

The need for molecular testing on tissue before treatment with selective *BRAF* inhibitors may substantially delay the time to initiation of a beneficial treatment in patients with *BRAF*–mutated ATC.<sup>8,18–22</sup> We have demonstrated previously



**Fig 1.** Patient selection and distribution. The study population was selected between March 2016 and July 2017; informed consent was obtained at the time of enrollment. ATC, anaplastic thyroid carcinoma; cfDNA, cell-free DNA, ddPCR, droplet digital polymerase chain reaction.

that *BRAF* V600E cfDNA can be detected in the blood of patients with ATC by a next-generation sequencing (NGS) platform with a faster turnaround time, which therefore serves as a surrogate for tissue biopsy.<sup>23</sup>

A study at our center has demonstrated that droplet digital polymerase chain reaction (ddPCR) can be used as a targeted analysis of cfDNA in the circulation of patients with medullary thyroid cancer and that changes in circulating tumor DNA levels correspond to treatment responses.<sup>24</sup> Therefore, we hypothesized that the detection of *BRAF*-mutated cfDNA by ddPCR will correlate with tissue-based molecular testing results and that changes in the level of the cfDNA will correlate with response to treatment in patients with ATC.

## MATERIALS AND METHODS

### Study Population for Testing for Concordance Between Circulating *BRAF* V600E cfDNA and Tumor Tissue and cfDNA by NGS

Patients with suspected ATC were prospectively enrolled between March 2016 and July 2017. Informed consent was obtained from the patients before collecting blood under an institutional review board protocol. All patients' tumor tissues were read by an independent head and neck pathologist at our center to confirm their diagnosis. Individuals who had unconfirmed ATC or no evidence of disease or who were being managed by observation for low

disease burden were excluded. Our standard of care is to obtain mutation tests on tumor tissue by NGS or immunohistochemistry (IHC) or cfDNA by NGS before initiation of treatment. In the absence of readily available tumor mutation profiling, we obtain both *BRAF* V600E by IHC on tissue biopsy and cfDNA on plasma by NGS simultaneously before initiation of treatment. Whenever feasible, these results are confirmed through testing on tumor tissue by NGS to validate treatment choice. The IHC analysis antibody is anti-*BRAF* p.V600E (clone VE1, dilution 1:50; Spring Bioscience, Pleasanton, CA) and is performed on a Ventana BenchMark ULTRA autostainer (Ventana Medical Systems, Tucson, AZ).

Patients who had a cfDNA analysis by NGS more than 2 weeks before the research ddPCR sample draw were excluded from concordance testing. However, if patients tested positive for the *BRAF* V600E mutation on their tumor tissue and were receiving treatment, serial plasma samples were collected for ddPCR assay during their treatment to examine the correlation of cfDNA levels with response to treatment.

### Study Population for Correlation of Changes in cfDNA Levels With Response to Treatment

Patients with tissue-positive *BRAF* V600E mutation were followed prospectively during their respective treatments to evaluate whether levels of circulating *BRAF* V600E cfDNA by ddPCR could serve as a biomarker for monitoring response to therapy. Blood samples for cfDNA analysis were drawn at routinely scheduled laboratory appointments.

### *BRAF* V600E Mutation Analysis

Tumor tissue was tested for *BRAF* mutation status by either IHC or NGS at our center (50-gene somatic mutation analysis panel,<sup>25</sup> Solid Tumor Genomics Assay version 1 that examines 134 genes,<sup>26</sup> or FoundationOne genomic test<sup>27</sup>), depending on the quantity of viable tumor tissue available for testing. Some patients had mutation testing by PCR before referral to our center. Blood samples were drawn for ddPCR testing as described in the Appendix and sent for cfDNA analysis by NGS using the Guardant360

**Table 1.** Patient Characteristics

Characteristic	No. (%)
No. of patients	44
Median age, years (range)	66 (47-89)
Sex	
Male	34 (77)
Female	10 (23)
Stage at diagnosis	
IVA	2 (5)
IVB	18 (40)
IVC	24 (55)
<i>BRAF</i> status on tumor	
<i>BRAF</i> V600E mutation*	20 (45)
<i>BRAF</i> wild type	24 (55)
Method of <i>BRAF</i> analysis on tumor	
NGS	39 (89)
IHC without NGS†	5 (11)
Baseline liquid biopsy sample by NGS	
Drawn	43 (98)
Not drawn	1 (2)

NOTE. NGS by Guardant360 kit (Guardant Health, Redwood City, CA) was used for cell-free DNA analysis on plasma samples. One patient did not have a liquid biopsy sent for NGS by Guardant360 at the time of data analysis.

Abbreviations: IHC, immunohistochemistry; NGS, next-generation sequencing.

\*Of these patients, 17 (85%) had tumor tissue tested by NGS and three (15%) by IHC.

†Of the five patients who were tested for the presence of the *BRAF* V600E protein by IHC, one had testing for and detection of the same mutation by single-gene polymerase chain reaction at an outside facility before referral to our center. Two patients who had tumor tissue tested only by IHC did not have additional testing by NGS because of insufficient tumor tissue.

sequencing test<sup>28</sup> (Guardant Health, Redwood City, CA).

### Evaluation of Response to Treatment

A single independent radiologist reviewed the images at baseline and at the time of restaging while patients were receiving treatment. We determined clinical responses by calculating the percent change (from baseline and after/during treatment) in the size of target lesions identified using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1,<sup>29</sup> but we modified slightly the definitions of response. We calculated the change in *BRAF*-mutated cfDNA level and correlated that with the change in the sum of the size of target lesions. For the purpose of our study, an increase in tumor size was defined as an increase in the sum of target lesions greater than or equal to 10 mm. RECIST progression in nontarget lesions was assigned an additional 20 mm to the sum of target lesions. A

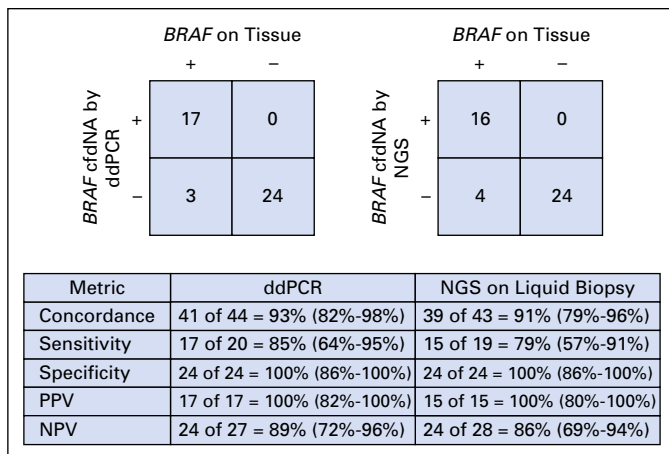
decrease in tumor size was defined as a decrease in sum of target lesions greater than or equal to 10 mm. Stable tumor size was defined as a change in tumor burden between 9 and -9 mm.

### Data Analysis

**Testing for concordance between circulating V600E cfDNA by ddPCR and tumor tissue and cfDNA by NGS and tumor tissue.** We defined a true-positive sample by the presence of *BRAF* V600E mutation in cfDNA (by either ddPCR or NGS) and on tissue testing. Similarly, true-negative samples had the absence of *BRAF* V600E mutation detection in both cfDNA and tissue testing. A false positive was defined by the presence of a mutation in cfDNA when tumor tissue tested negative, and a false negative was indicated when the mutation was not detected in cfDNA but tumor tissue tested positive for *BRAF* V600E mutation.

Concordance between *BRAF* V600E mutation in cfDNA by ddPCR with *BRAF* mutation status on tumor tissue by NGS, IHC, and PCR was calculated as number of concordant samples / (number of concordant samples + number of discordant samples). Concordance also was calculated for the detection of *BRAF* mutation in cfDNA samples by NGS and tumor tissue.

**Testing for correlation of change in circulating V600E ddPCR levels with changes in tumor size.** In patients who were followed prospectively, concordance was calculated between the change in the level of *BRAF*-mutated cfDNA by ddPCR and change in tumor size on restaging scans during treatment. Any increase in the level of cfDNA was called concordant if that correlated with an increase in tumor size of greater than or equal to 10 mm. Similarly, any decrease in the level of cfDNA was called concordant if it correlated with a decrease in tumor size of greater than or equal to 10 mm. If there was no change in the level of cfDNA correlating with 9 to -9 mm, the sample was called concordant. If the changes in the levels of cfDNA did not meet these three criteria, they were called discordant. Samples drawn in between restaging scans were studied in correlation with the change in tumor size on follow-up imaging to evaluate whether the test was predictive of response to therapy.



**Fig 2.** Concordance between detection of circulating *BRAF* V600E cell-free DNA (cfDNA) by droplet digital polymerase chain reaction (ddPCR) and next-generation sequencing (NGS) on liquid biopsy with mutation status on tumor. Differences in the ability to detect and concordance with *BRAF* positivity on tumor tissue are shown. One patient had cfDNA tested by ddPCR, but no sample was drawn simultaneously for NGS on liquid biopsy. Confidence intervals are shown in parentheses. NPV, negative predictive value; PPV, positive predictive value.

### Statistical Tests

Sensitivity and specificity of detection of *BRAF*-mutated cfDNA by ddPCR and by NGS were calculated and reported with exact 95% CIs. Changes between levels of circulating cfDNA and change in the sum of target lesions at the time of each restaging were studied using Spearman rank correlation coefficient. Fisher's exact test was conducted to calculate concordance between cfDNA levels and the corresponding radiologic events, specifically, increase in tumor size, tumor shrinkage, and stable tumor size.

## RESULTS

Fifty-eight patients with a suspected ATC diagnosis were prospectively enrolled in the study. Fourteen were excluded (Fig 1). Of the remaining 44 patients, 16 who had a *BRAF* V600E-positive tumor were followed for correlation changes in cfDNA with response to treatment.

### Patient Characteristics

Patient characteristics are listed in Table 1. Median age was 66 years (range, 47 to 89 years). The majority were men (77%). At the time of diagnosis, 24 patients (55%) had stage IVC disease. Tumor somatic mutation testing was performed by NGS in 39 patients (89%), with the remaining patients evaluated by *BRAF* mutation-specific IHC. Twenty patients (45%) were positive for *BRAF* V600E mutation. Forty-three (98%) also had blood samples sent for cfDNA by NGS.

### Detection of *BRAF* V600E cfDNA on Liquid Biopsy

The lowest allelic fraction (AF) for *BRAF* V600E detected by ddPCR was 0.07% and for cfDNA by NGS, 0.1%. The median AF was 0.42% (range, 0.07% to 20.9%) and 1.1% (range, 0.1% to 26.4%) by ddPCR and NGS assays, respectively. Of the 20 patients who tested positive for *BRAF* mutation on tissue, 17 (85%) were positive for *BRAF* V600E cfDNA by ddPCR and 16 (80%) were positive for cfDNA by NGS (Fig 2). One patient was true positive for cfDNA by NGS but false negative by ddPCR. Two patients with *BRAF*-mutated tumor tissue tested negative for cfDNA by NGS but tested positive by ddPCR. An additional two patients with *BRAF* V600E-positive tumor tissue were negative by both liquid biopsy approaches. Overall, three patients tested as false negative by ddPCR cfDNA analysis. All 24 patients whose tumor tissue tested negative for *BRAF* V600E were also negative for cfDNA by NGS and ddPCR liquid biopsy. The sensitivity of *BRAF* V600E cfDNA detection was 85% for ddPCR and 79% for NGS. Thus, a 6% difference was found in the sensitivity of the two tests (95% CI, -18% to 29%). The specificity of both liquid biopsy tests for the detection of *BRAF* V600E cfDNA was 100%. Figure 3 shows the receiver operating characteristic curve for this data set. The two data sets produced identical results, so only a single curve is shown (area under the curve, 0.90; 95% CI, 0.80 to 0.99; Wilcoxon rank sum test  $P < .001$ ). Detection of *BRAF*-mutated cfDNA by ddPCR was 93% concordant with tumor testing, whereas detection of cfDNA by NGS was 91% (95% CI, -8% to 14%). Both liquid biopsy assays were found to be similar in accuracy and concordance.

### Correlation Between Changes in *BRAF* V600E cfDNA by ddPCR and Changes in Tumor Size

Sixteen patients whose tumors tested positive for *BRAF* mutation were prospectively followed and had plasma samples drawn during scheduled laboratory appointments before the clinic visit. The details of these patients are listed in Table 2. Median time between the samples was 3.83 weeks (range, 1.3 to 18 weeks). Median staging interval for scans was 6 weeks (range, 1.6 to 20.4 weeks). Study patients underwent one to eight

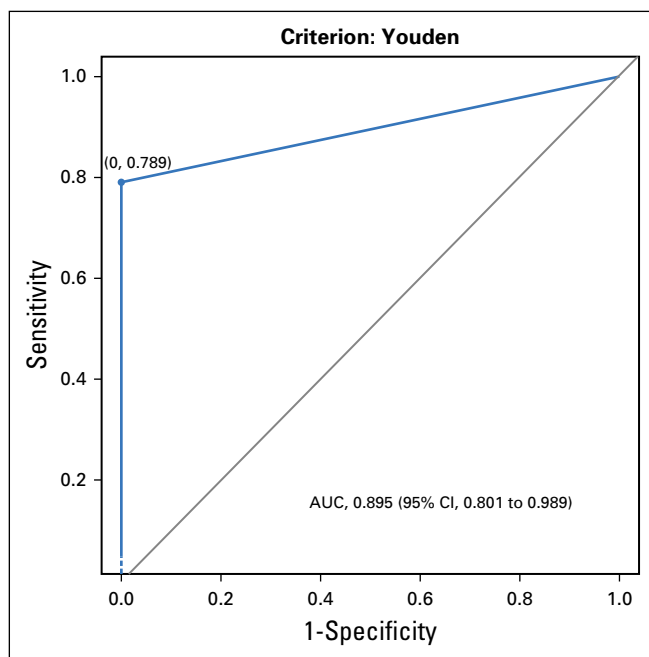
post-treatment scans (median, 3.5 scans) and were exposed to one to three treatments. Thirty-six radiologic assessments were available for direct comparison with plasma samples. At the time of baseline cfDNA sampling, the median tumor burden by imaging was 76.8 mm (range, 8 to 146.9 mm). *BRAF* V600E cfDNA was detected in 13 of 16 patients. In the three patients who tested false negative by ddPCR, tumor burden at baseline ranged from 31 to 33.3 mm. These patients had had their primary tumor treated with surgery (thyroidectomy in two patients and hemithyroidectomy in one patient). The *BRAF* V600E cfDNA was detectable in tumors larger than 52.5 mm, with the exception of a single patient who had an 8-mm lung nodule that presented while he was receiving adjuvant radiation to the thyroid bed with chemotherapy.

Of 36 time points, 27 (75%) provided concordant findings between changes in cfDNA levels and response to therapy (Table 3). Seventeen scans had findings consistent with decreases in tumor size, of which 16 (94%) were concordant with reductions in *BRAF* V600E cfDNA. We included the three patients who tested false negative by ddPCR in this group because the *BRAF* V600E cfDNA levels continued to remain undetectable, which correlated with these patients' continued response to treatment with *BRAF* inhibitor. Increased tumor size was observed in 15 scans, of which seven (47%) were concordant with increased cfDNA levels. Stable tumor size

was observed for four restaging scans, which correlated with no change in cfDNA levels. Overall comparison of response determined by imaging versus levels of cfDNA by ddPCR was statistically significant when analyzed using Fisher's exact test ( $P = .0061$ ). On analyzing the change in levels of cfDNA with changes in tumor size, the Spearman rank correlation coefficient was 0.37 (95% CI, 0.05 to 0.62;  $P = .029$ ).

Eleven patients (65%) had a total of 17 samples drawn between restaging scans. Of these, 12 (71%) revealed a change in cfDNA level predictive of the image-based change in tumor size. The median time between treatment start and the next sample draw was 2.9 weeks (range, 0.14 to 5.9 weeks). The earliest predictor of response was seen at 1 day after surgery and 2 weeks after starting *BRAF* inhibitor therapy. One patient showed an increase in cfDNA levels 2 weeks after starting *BRAF* inhibitor therapy, which was consistent with the increased tumor size observed on his first restaging scans 2 months after starting this treatment. The median time between a sample and follow-up imaging was 3.7 weeks (range, 1.14 to 3.9 weeks). Figure A1 shows an example of the trends in cfDNA levels that correlated with changes in tumor size on imaging in a patient who was initially treated with nab-paclitaxel followed by *BRAF* and MEK inhibitor therapy and then with the addition of immunotherapy (pembrolizumab). The levels of *BRAF* cfDNA trended upward despite the addition of pembrolizumab because the patient was not able to swallow the *BRAF* inhibitor, which led to nonadherence and eventual progression.

**Fig 3.** Receiver operator characteristic curve. One patient with *BRAF* positivity on tumor did not get testing done by next-generation sequencing (NGS) on liquid biopsy; therefore, the analysis was done on 43 patient samples. Because all 24 of the patients without *BRAF* mutation tested negative for cell-free DNA by droplet digital polymerase chain reaction (ddPCR) and NGS, the receiver operating characteristic curve is degenerate. On cell-free DNA testing by NGS, three of 20 patients with *BRAF* positivity on tumor tissue tested negative. On ddPCR, four of 19 patients with *BRAF* positivity on tumor tissue tested negative. The plots and areas under the curve (AUCs) are identical for ddPCR and NGS on liquid biopsy. Wilcoxon rank sum test  $P < .001$ .



## DISCUSSION

The *BRAF* V600E mutation is the most common actionable mutation in ATC tumors. Recent studies have shown promising results with dabrafenib plus trametinib (*BRAF* and MEK inhibitors) in terms of clinical response and survival,<sup>9,18,30</sup> and this combination is now Food and Drug Administration approved for *BRAF*-mutated ATC. Hence, identification of this mutation for timely initiation of treatment is important.

In addition to the ability to identify an actionable mutation for initiation of therapy, development of biomarkers is needed to predict and assess treatment response in ATC. In differentiated thyroid cancer (DTC), serum thyroglobulin

**Table 2.** Characteristics of Patients With *BRAF* Mutation on Liquid Biopsy Samples

Characteristic	No. (%)
No. of patients	16
Median age, years (range)	68 (46-81)
Sex	
Male	10 (63)
Female	6 (37)
Stage at diagnosis	
IVA	0 (0)
IVB	6 (37)
IVC	10 (63)
Treatment received*	
Surgery	2 (12)
Radiation ± sensitizing chemotherapy	4 (25)
Systemic therapy	5 (31)
BRAF inhibitor + MEK inhibitor	12 (75)
BRAF inhibitor + MEK inhibitor + immunotherapy	4 (25)

NOTE. Patients with a *BRAF* mutation on their tumor tissue had liquid biopsy samples drawn prospectively for assessing correlation of changes in levels of circulating *BRAF* V600E cell-free DNA detected by droplet digital polymerase chain reaction with response to treatment.

\*Patients received more than one treatment modality.

(Tg) levels are a biomarker of response to therapy. In ATC, Tg measurements play a small role because the dedifferentiation associated with these tumors generally results in loss of Tg expression. Given the successes observed for other cancers where tumor-specific biomarkers do not exist, we believe that changes in *BRAF* V600E cfDNA could provide a specific biomarker for monitoring response to treatment in patients with ATC.<sup>19</sup> Although response can be assessed by imaging, a marker to predict response allows sufficient time to change the treatment plan before the patient becomes too ill.

In this study, we show that cfDNA by ddPCR is a sensitive and highly specific technique for determining *BRAF* status in patients with ATC. This test was found to have a concordance similar to cfDNA by NGS in relation to the *BRAF* status on the tumor. *BRAF* cfDNA and RNA in liquid biopsy have been shown to be useful biomarkers in patients with DTC as well as in a murine model of ATC.<sup>31,32</sup> The current study is the first in our knowledge to evaluate ddPCR to detect *BRAF*-mutated cfDNA in the circulation of patients with ATC. In a phase II clinical trial in radioiodine-refractory DTC, *BRAF* V600E cfDNA was detectable by ddPCR in only 35% of patients,<sup>33</sup> whereas in our study, it was detected in 85% of patients with ATC. The release of tumor DNA into the circulation is believed to be a direct measure of tumor cell death. Unlike DTC tumors, greater cellular turnover occurs in ATC tumors, which leads to greater shedding of genetic material into the circulation. However, even with high tumor cell turnover, tumor burden may not be sufficient to permit the detection of circulating tumor DNA. In the current study cohort, ddPCR failed to detect mutation in three patients with *BRAF* V600E-mutated tumors. The inability to detect mutation could be explained by the low overall disease burden on imaging compared with positive patients. Therefore, we suggest that in patients with low measurable disease, reliance solely on cfDNA for identifying the *BRAF* mutation may not be advisable, and molecular testing on tumor tissue should be awaited.

A murine model of *BRAF*-mutated ATC circulating RNA levels has been shown to be a useful biomarker of response to therapy.<sup>32</sup> In the current study, we show that ddPCR is a useful technique for serial monitoring of cfDNA levels as a predictive biomarker in patients with ATC

**Table 3.** Concordance of Changes in cfDNA With Changes in Tumor Burden at Restaging

<i>BRAF</i> cfDNA by ddPCR	Radiographic Determination, No. (%)		
	Increase in Tumor Size	Decrease in Tumor Size	Stable Tumor Size
No. of patients	15	17	4
Concordant with changes in tumor size	7 (47)	16 (94)	4 (100)
Discordant with changes in tumor size	8 (53)	1 (6)	0 (0)

NOTE. Increase in tumor size was defined as any change in the sum of target lesions ≥ 10 mm. Progression in nontarget lesions (ie, nonmeasurable disease) was assigned an additional 20 mm to the sum of target lesions. Decrease in tumor size was defined as any reduction in sum of target lesions ≥ 10 mm. Stable tumor size was defined as any change in tumor burden between 9 and -9 mm. *P* = .0061 (Fisher's exact test).

Abbreviations: cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction.

on treatment. Changes in levels of cfDNA were seen as early as 1 day after surgery and 2 weeks after starting BRAF inhibitor therapy, consistent with tumor shrinkage. The changes in cfDNA levels were concordant with changes in tumor size on imaging. These findings were similar to that observed in patients with melanoma.<sup>34,35</sup> In patients whose *BRAF* V600E cfDNA levels rise after initiating BRAF and MEK inhibitor therapy, resistance to treatment should be considered, prompting early restaging scans and consideration of alternative treatment options upon confirmation. Our findings also highlight the limitation of using a single-gene cfDNA as a biomarker in a heterogeneous tumor such as ATC. In patients who experience an increase in tumor size, fewer than one half showed the expected concomitant increases in *BRAF* V600E cfDNA. One possible explanation is that targeted treatment indeed led to a reduction in the *BRAF* V600E-harboring tumor cells but led to clonal expansion of tumor cells that harbored other driver mutations as a mechanism of

resistance. Such a possibility could be addressed through tumor biopsy or cfDNA by NGS to look for the emergence of new mutations.

Our results strongly support the use of ddPCR for identifying the *BRAF* V600E mutation in patients with ATC. However, the small sample size limited us from evaluating the association of the AF of the *BRAF* V600E cfDNA with prognosis and overall survival.

In conclusion, ddPCR is a novel, noninvasive technique for detecting *BRAF* V600E cfDNA in patients with ATC and should be considered in routine standard-of-care testing. The results show that cfDNA potentially could serve as a biomarker to predict response in patients with ATC before restaging scans, which is important in the management of an aggressive cancer like ATC where timely treatment is crucial for prolonging survival.

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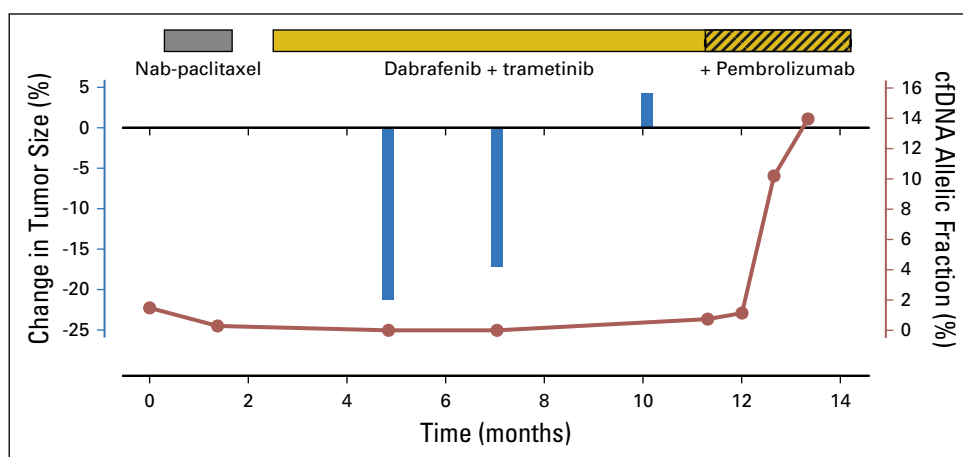


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## Appendix

**Fig A1.** Trends in cell-free DNA (cfDNA) levels that correlate with changes in tumor size on imaging. cfDNA declined at the time of response to therapy correlated with tumor shrinkage from baseline, and an increase in cfDNA levels at the time of progression correlated with a radiologic increase in tumor burden.



### Isolation of Cell-Free DNA for Droplet Digital Polymerase Chain Reaction

After obtaining patient consent, approximately 8 to 10 mL of blood were drawn into each of two EDTA tubes. Plasma was separated from the cell components after centrifuging twice at 2,000 rpm for 10 minutes at 4°C and then stored at -80°C to allow for batch isolation of cell-free DNA (cfDNA). Before cfDNA isolation, plasma was thawed on ice and then centrifuged again to remove particulate. QIAmp Circulating Nucleic Acid Kit (QIAGEN, Venlo, the Netherlands) was used to isolate cfDNA from plasma. A standardized plasma volume of 3 mL was adapted for each sample, with a 50 µL elution volume. The amount of DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA).

### Droplet Digital Polymerase Chain Reaction

The detection of the *BRAF* V600E mutation was performed in a research laboratory setting using the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, CA) and the validated PrimePCR ddPCR Mutation Assay (Bio-Rad), which was multiplexed with an assay for detection of *BRAF* unmutated codon (wild type). Each reaction contained 11 µL of 2× Supermix, 1.1 µL of 20× Assay 1 HEX (wild-type allele), and 1.1 µL of 20× Assay 2 FAM (mutant allele) per reaction for a total of 13.2 µL per reaction. The remaining 8.8 µL contained cfDNA sample. The cfDNA yields using this protocol ranged from 2 to 132 ng/mL when measured by Qubit fluorometer, which allowed a median estimated input of 5 ng per assay. A nontemplate control of water was run for each assay as well as a *BRAF* V600E positive control (KTC1 cell line DNA). Droplets were counted with the QX200 Droplet Reader, and data were analyzed using QuantaSoft software (Bio-Rad), with thresholds for FAM and HEX detection manually set on the basis of results from the no-template control. We required a minimum of 8,000 counted droplets. The detection of *BRAF* V600E was considered positive if two or more positive droplets were detected in a single assay. The allelic fraction was calculated, with the ratio of channel 1 counts (*BRAF* V600E cfDNA) over channels 1 and 2 (*BRAF* V600E and *BRAF* WT) and the value standardized as a percentage.