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Detection of Cancer DNA in Plasma of Patients with Early-Stage Breast Cancer

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Abstract

Purpose: Detecting circulating plasma tumor DNA (ptDNA) in patients with early-stage cancer has the potential to change how oncologists recommend systemic therapies for solid tumors after surgery. Droplet digital polymerase chain reaction (ddPCR) is a novel sensitive and specific platform for mutation detection.

Experimental Design: In this prospective study, primary breast tumors and matched pre- and post-surgery blood samples were collected from patients with early-stage breast cancer ($n = 29$). Tumors ($n = 30$) were analyzed by Sanger sequencing for common *PIK3CA* mutations, and DNA from these tumors and matched plasma were then analyzed for *PIK3CA* mutations using ddPCR.

Results: Sequencing of tumors identified seven *PIK3CA* exon 20 mutations (H1047R) and three exon 9 mutations (E545K). Analysis of tumors by ddPCR confirmed these mutations and identified five additional mutations. Presurgery plasma samples ($n = 29$) were then analyzed for *PIK3CA* mutations using ddPCR. Of the 15 *PIK3CA* mutations detected in tumors by ddPCR, 14 of the corresponding mutations were detected in presurgical ptDNA, whereas no mutations were found in plasma from patients with *PIK3CA* wild-type tumors (sensitivity 93.3%, specificity 100%). Ten patients with mutation-positive ptDNA presurgery had ddPCR analysis of postsurgery plasma, with five patients having detectable ptDNA postsurgery.

Conclusions: This prospective study demonstrates accurate mutation detection in tumor tissues using ddPCR, and that ptDNA can be detected in blood before and after surgery in patients with early-stage breast cancer. Future studies can now address whether ptDNA detected after surgery identifies patients at risk for recurrence, which could guide chemotherapy decisions for individual patients. *Clin Cancer Res; 20(10); 2643–50. ©2014 AACR.*

Introduction

The goal of adjuvant systemic therapies in clinical oncology, such as chemotherapy administered after breast cancer surgery, is to eradicate microscopic residual disease that could lead to recurrence and incurable disease. Large randomized prospective trials support the use of adjuvant systemic therapies, however these trials also show the majority of patients (~60%–70%) with early-stage breast cancer are cured with local therapies alone and that the addition of adjuvant therapies improves disease-free survival and cure rates by ~10% to 20% (1). Although this is a relatively small percentage of patients, this equates to tens of thousands of lives that are cured because of the administration of additional systemic therapies. However, it is also known that many systemic therapies have toxicities such as infection, neuropathy, heart failure, and secondary leukemias from chemotherapies that can even lead to premature deaths. Because there is no reliable method for detecting microscopic residual disease after surgery, the current paradigm is to recommend adjuvant therapies to the majority of patients to benefit relatively few, resulting in overtreatment. The ability to develop accurate markers of microscopic residual disease after surgery could identify patients who are at higher versus lower risk for recurrence, and potentially spare patients toxic therapies that are not needed. This could lead to a paradigm shift of individualized adjuvant therapy recommendations based on the presence or absence of residual disease postsurgery.

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Translational Relevance

In this study, the feasibility and accuracy of plasma tumor DNA detection was prospectively assessed by screening for the presence of tumor *PIK3CA* mutations in patients' plasma before and after breast surgery using droplet digital PCR (ddPCR), a second-generation digital PCR platform. We observed in patients with early-stage breast cancer that ddPCR could detect *PIK3CA* mutations preoperatively with 93.3% sensitivity (14/15 mutations detected) and 100% specificity (no false positives in 30 tumors). Moreover, 5 of 10 patients with *PIK3CA* mutation-positive tumors continued to have mutant *PIK3CA* detected in their postoperative blood sample despite having no clinical evidence of disease. This work is the first to demonstrate in a prospective fashion that ddPCR can be used to accurately detect cancer DNA mutations in the blood of patients with early-stage breast cancer, including those with minimal, clinically undetectable disease. Our results suggest that ddPCR provides an accurate and relatively noninvasive method for measuring residual disease and could be developed as a biomarker to identify patients with early-stage breast cancer at higher risk for recurrence to help make informed decisions about adjuvant therapies.

During the past few years, studies have demonstrated the ability to reliably detect and quantify circulating cell-free cancer DNA and RNA, mostly in patients with metastatic disease (2–10). This is predicated on the principle that cancer DNA contains somatic mutations, that is, DNA alterations that are unique to cancer cells and not present in normal cells. Circulating cell-free DNA is thought to be shed or released from normal and cancer cells (11, 12). For clarity and precise nomenclature, we will refer to circulating cell-free plasma tumor DNA as plasma tumor DNA (ptDNA) because circulating tumor DNA also refers to urine tumor DNA and tumor DNA in other bodily fluids. The ability to accurately detect the small proportion of ptDNA that exists within the larger population of noncancerous-derived plasma DNA holds great promise for guiding systemic therapy decisions in oncology (13). For example, using blood as a "liquid biopsy," one can noninvasively identify specific mutations within a patient's cancer (14). In addition, because of the quantitative nature of ptDNA detection and the short half-life of plasma DNA (1–2 hours), detection of minimal tumor burden and monitoring response to therapies in metastatic disease has been shown to be feasible (4, 8).

Technologic advances have raised the possibility of incorporating ptDNA testing for use in clinical oncology. Specifically, digital PCR and next-generation sequencing allow for the detection of mutant DNA molecules that are present at 0.02% to 0.1% of the total amount of DNA assayed (2, 3). In essence, these technologies partition individual DNA molecules and then each molecule is amplified and queried for a given mutation (15). This dramatically increases the ability to detect rare mutant DNA molecules among a vast population of normal or wild-type DNA. Prior studies have been conducted mostly in the metastatic setting where the amount of total plasma DNA and ptDNA tends to be greater, and sensitivity for detecting ptDNA is generally not an issue (2, 4). For example, using varying techniques studies have reported detection of ptDNA in patients with metastatic breast cancer with high sensitivities and specificities (5, 7). In addition, a recent study has demonstrated the ability to detect miRNAs in metastatic prostate cancer using digital PCR (10). Moreover, a recent prospective study in patients with metastatic breast cancer compared ptDNA to circulating tumor cells and the breast cancer serum marker CA 15–3, and demonstrated ptDNA more accurately reflects tumor burden with a greater dynamic range for following response to therapies and disease progression (2). However, only a few studies have evaluated ptDNA in patients with early-stage cancer, and in these analyses the ability to identify ptDNA has varied, likely because of the technical limits of these methods to detect smaller tumor burden (9, 14, 16–19). Indeed, these studies suggest that more sensitive methods are needed to utilize ptDNA as a cancer biomarker for early-stage disease.

Here we describe a prospective study in which we examined the ability to detect mutant *PIK3CA* molecules in ptDNA from patients with early-stage (stage I–III) breast cancer using next-generation digital PCR platforms. *PIK3CA* is an oncogene that encodes the p110α component of phosphoinositide 3-kinase (PI3K), and there is currently intense interest to develop PI3K inhibitors because of the high frequency of *PIK3CA* mutations in human cancers (20). There are 3 frequently recurring "hotspot" *PIK3CA* mutations within 2 exons (exon 9: E542K and E545K and exon 20: H1047R), which account for 80% to 90% of all *PIK3CA* mutations found in human malignancies (21). Multiple cancer sequencing studies have found *PIK3CA* mutations to be present in ∼30% of all breast cancers, with a higher frequency (∼45%) reported in estrogen receptor/progesterone receptor (ER/PR)–positive and human epidermal growth factor receptor 2 (HER2)–positive breast cancers (22–25). In prior work, we and others demonstrated that mutant *PIK3CA* DNA can be reliably detected in plasma from patients with metastatic breast cancer using various technologies (2, 5, 16). We hypothesized that the newer technique of droplet digital PCR (ddPCR) could identify *PIK3CA* mutations in formalin fixed paraffin embedded (FFPE) primary tumor samples and corresponding plasma from patients with early--stage breast cancer before and after surgery with high sensitivity and specificity. If feasible, this would allow for future trials testing the clinical utility of ptDNA as a cancer biomarker to guide individual decisions about adjuvant systemic therapies.

Materials and Methods

Patients and sample collection

For each patient, a single presurgery blood sample was collected before definitive surgery, either lumpectomy or

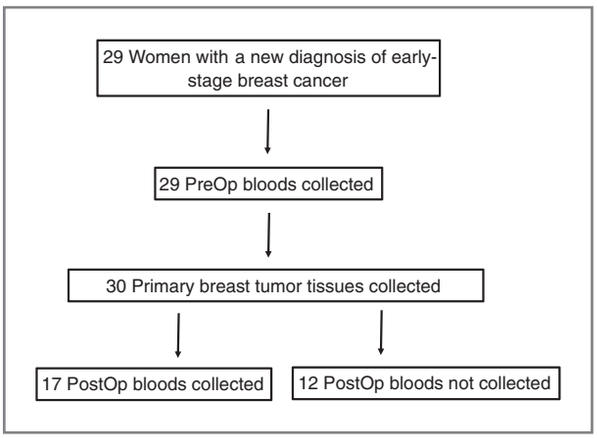

Figure 1. Enrollment of patients and collection of clinical samples. A total of 29 women were enrolled in this prospective study. *PIK3CA* mutational analysis was performed on 30 tumor specimens and pre- and postsurgery plasma samples collected. In 12 patients, postsurgery blood samples could not be collected. PreOp denotes presurgery specimens and PostOp denotes postsurgery specimens.

mastectomy. In addition, we collected a second blood sample after surgery. Ten milliliters (10 mL) of blood was collected at each time point. Primary tumor samples were obtained as FFPE blocks and slides prepared for DNA analysis as previously described (ref. 5; see also Supplementary Methods). Of the 29 patients, all had primary cancer tissue and presurgery blood collected, whereas 17 patients had postsurgery blood collected (Fig. 1).

Tissue DNA sequencing and ddPCR of FFPE samples

Genomic DNA was extracted from tumor and adjacent normal tissues, and purified as previously described (ref. 5; see also Supplementary Methods). PCR primers used for amplifying segments of *PIK3CA* exon 9 and exon 20, and the nested sequencing primers used for Sanger sequencing are shown in Supplementary Table S1. Genomic DNA was also analyzed by ddPCR using *Taq*Man probes for wild-type *PIK3CA*, as well as *PIK3CA* E545K and *PIK3CA* H1047R mutations to detect and quantitate these mutations (Supplementary Table S2). ddPCR results were quantified using RainDrop Analyst software (RainDance Technologies) and are expressed as a percentage or fractional abundance of mutant to total (mutant plus wild type) *PIK3CA* molecules for each sample (see also Supplementary Methods).

Isolation and quantification of ptDNA by ddPCR

Blood samples and plasma DNA preparation were performed as previously described (ref. 5; see also Supplementary Methods). Purified plasma DNA was subjected to high-fidelity PCR amplification using the primers listed in Supplementary Table S3. The PCR amplified products were then diluted and combined with mutant and wild-type probes for *PIK3CA* E545K and *PIK3CA* H1047R mutation detection in separate reactions for each mutation specific probe (Supplementary Table S3). ddPCR was then performed per the manufacturer's protocol with results reported as a percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild type) DNA alleles.

Statistical analysis

This was a feasibility study to determine the accuracy of detecting *PIK3CA* mutations using ddPCR on presurgery plasma from patients with early-stage breast cancer. We estimated that for 95% accuracy with a 95% lower limit confidence interval of 80%, 30 patient samples with approximately half containing *PIK3CA* mutations would be sufficient. Using the ddPCR results from the FFPE analysis as our reference, Java Stat 2-way contingency tables were used to calculate sensitivity, specificity, accuracy, and 95% confidence intervals (CI) for the detection of ptDNA in the presurgery plasma samples taking into account both *PIK3CA* E545K and H1047R assays.

To quantify the percent ptDNA containing mutant *PIK3CA* in plasma samples, a fractional abundance calculation using the QuantaSoft program (Bio-Rad Technologies) was used, using the total number of droplets (with and without DNA) to calculate the number of DNA molecules as copies/μL, and then dividing the number of mutant DNA molecules by the number of total DNA molecules (mutant plus wild type), multiplied by 100 to yield a percentage of mutant DNA molecules in a sample taking into account a Poisson distribution of occupied to unoccupied droplets (10). In addition, this program was used for the summation of each category of droplets in multiple replicates creating a single meta-well for each sample with a 95% CI calculated using Poisson statistics as previously described (10).

Results

Twenty-nine patients with early-stage (stage I–III) breast cancer were consented and enrolled between August 2010 and January 2011 in a prospective Institutional Review Board–approved study at Johns Hopkins. Patient characteristics are described in Table 1. The median age of enrolled patients was 60. Although not preenriched, the majority of patients (93%) had ER/PR–positive disease. One patient (patient 14) had bilateral breast cancers at diagnosis. At the time of data analyses, the median duration of follow up for all 29 patients was 36 months and 2 patients had recurred.

PIK3CA mutation detection in FFPE samples with ddPCR

Tumor specimens with corresponding clinical and pathologic information were collected from all patients. Sanger sequencing of these 30 primary tumor specimens and adjacent normal tissues (controls) from 29 patients identified 7 *PIK3CA* exon 20 mutations (H1047R) and 3 exon 9 mutations (E545K) yielding a frequency of 33.3% (10 of 30 tumors). No other mutations within these coding regions were found. These DNA samples were then subjected to ddPCR, which confirmed the 10 mutations identified by Sanger sequencing. Mutant to wild-type *PIK3CA* fractional abundance ranged from 13.8% to 55.6%. ddPCR also

Table 1. Patient characteristics

Patient	Age at diagnosis (media, $n = 60$)	Stage	TNM	Histology	ER	PR	HER2
1	66	IA	T1c N0(i+) Mx	Lobular	Positive	Positive	Negative
2	63	IIA	T1b N1 Mx	Ductal	Positive	Positive	Negative
3	52	IA	T1c N0 Mx	Ductal/lobular	Positive	Positive	Negative
4	77	IA	T1b N0 Mx	Lobular	Positive	Positive	Negative
5	48	IA	T1c N0 Mx	Ductal	Negative	Negative	Negative
6	38	IIB	T2 N1 Mx	Ductal	Positive	Positive	Negative
7	49	IIA	T2 N0 Mx	Ductal	Positive	Positive	Negative
8	51	IA	T1c N0 M0	Ductal	Positive	Positive	Negative
9	48	IIA	T2 N0 Mx	Ductal	Positive	Positive	Negative
10	55	IA	T1c N0 M0	Ductal	Positive	Positive	Negative
11	71	IA	T1c N0 Mx	Ductal	Positive	Negative	Negative
12	69	IIA	T2 N0(i-) Mx	Lobular	Positive	Positive	Negative
13	52	IA	T1c N0 Mx	Ductal/lobular	Positive	Positive	Negative
14	64	IIA	R: T1a N1 Mx	Ductal	Positive	Positive	Negative
		IIIA	L: T1b N2 Mx	Lobular	Positive	Positive	Negative
15	75	IA	T1b N0 Mx	Lobular	Positive	Positive	Negative
16	51	IIB	T2 N1 MX	Metaplastic	Negative	Negative	Negative
17	67	IA	T1c N0 Mx	Ductal/lobular	Positive	Positive	Negative
18	63	IA	T1c N0 Mx	Ductal	Positive	Positive	Negative
19	60	IIA	T1c N1 Mx	Ductal	Positive	Positive	Negative
20	38	IIB	T2 N1 Mx	Ductal	Positive	Positive	Negative
21	72	IIA	T2 N0 Mx	Ductal	Positive	Positive	Negative
22	59	IA	T1c N0 M0	Ductal	Positive	Positive	Negative
23	53	IIIA	T2 N2 Mx	Ductal	Positive	Positive	Negative
24	67	IA	T1c N0 Mx	Ductal/lobular	Positive	Positive	Negative
25	54	IA	T1c N0 Mx	Ductal	Positive	Positive	Negative
26	58	IIB	T2 N1 MX	Ductal	Positive	Positive	Negative
27	63	IIA	T1 N1 Mx	Ductal	Positive	Positive	Negative
28	55	IIA	T1c N1 Mx	Ductal	Positive	Positive	Negative
29	61	IIB	T2 N1mi Mx	Ductal	Positive	Positive	Equivocal[a]

NOTE: $n = 29$. TNM, tumor–node–metastasis.

[a]Equivocal by immunohistochemical staining 2+, and FISH ratio of HER2:chromosome 17 = 2.1.

identified 2 additional *PIK3CA* H1047R mutations, 1 additional *PIK3CA* E545K mutation and 1 sample with both H1047R and E545K mutations. The prevalence of *PIK3CA* mutations detected per tumor by ddPCR was 46.7% (14 of 30). Results comparing the FFPE and ddPCR analyses with fractional abundance are shown in Table 2.

PIK3CA mutations detected in ptDNA by ddPCR before surgery

Matched presurgery plasma samples were collected for all patients ($n = 29$). The amount of total plasma DNA varied among samples ranging from 3.07 to 22.60 nanograms, which is consistent with prior reports (26–29). These presurgery plasma samples were analyzed for *PIK3CA* E545K and H1047R mutations using ddPCR for detection. ddPCR detected 4 patients with *PIK3CA* exon 9 mutations in their presurgery ptDNA with a fractional mutation abundance ranging from 0.01% to 0.07%. Ten presurgery plasma samples were also identified as harboring a *PIK3CA* H1047R mutation with a fractional abundance ranging from 0.01% to 2.99%. Comparison of the ddPCR ptDNA results with corresponding ddPCR FFPE samples demonstrated that of the 15 *PIK3CA* mutations detected in tissue samples, identical mutations were detected in 14 of the matched ptDNA samples (Table 2). Notably, the single patient (patient 13) in whom the *PIK3CA* mutation was not detected in ptDNA had a low percentage of mutant *PIK3CA* in the tumor sample (not detected by Sanger sequencing) and a small amount of total plasma DNA (~5 ng). Importantly, none of the patients with *PIK3CA* wild-type tumors had detectable *PIK3CA* mutations in presurgery plasma DNA. These data demonstrate a sensitivity of 93.3% (95% CI, 75.5%–93.3%), specificity of 100% (95% CI, 82.2%–100.0%), and accuracy of 96.7% (95% CI, 78.9%–96.7%) of ddPCR for detecting mutant *PIK3CA* in presurgery ptDNA.

Table 2. Analysis of *PIK3CA* mutations in tumors and blood

Patient	Sequencing FFPE tumor tissue	ddPCR FFPE tumor tissue	ddPCR presurgery plasma
1	Wild type	H1047R (9.4%)	H1047R (0.03%)
2	Wild type	Wild type	Wild type
3	Wild type	Wild type	Wild type
4	Wild type	E545K (28.9%)	E545K (0.01%)
		H1047R (6%)	H1047R (0.02%)
5	H1047R	H1047R (25.5%)	H1047R (0.02%)
6	Wild type	Wild type	Wild type
7	Wild type	Wild type	Wild type
8	H1047R	H1047R (26%)	H1047R (0.12%)
9	E545K	E545K (43.7%)	E545K (0.02%)
10	Wild type	Wild type	Wild type
11	H1047R	H1047R (13.8%)	H1047R (0.01%)
12	Wild type	Wild type	Wild type
13	Wild type	E545K (16.1%)	Wild type
14	R:Wild type	Wild type	Wild type
	L:Wild type	Wild type	Wild type
15	Wild type	Wild type	Wild type
16	H1047R	H1047R (32.2%)	H1047R (2.99%)
17	E545K	E545K (55.6%)	E545K (0.07%)
18	Wild type	Wild type	Wild type
19	E545K	E545K (20.3%)	E545K (0.01%)
20	Wild type	Wild type	Wild type
21	Wild type	Wild type	Wild type
22	H1047R	H1047R (28.9%)	H1047R (0.02%)
23	Wild type	Wild type	Wild type
24	Wild type	Wild type	Wild type
25	H1047R	H1047R (30.5%)	H1047R (0.01%)
26	Wild type	Wild type	Wild type
27	Wild type	Wild type	Wild type
28	Wild type	H1047R (9.5%)	H1047R (0.02%)
29	H1047R	H1047R (43.1%)	H1047R (0.01%)

NOTE: Tumor and blood samples from 29 patients with early-stage breast cancer were analyzed for *PIK3CA* mutations using Sanger sequencing FFPE tumor tissue, ddPCR of FFPE tumors (ddPCR FFPE tumor tissue) and presurgery plasma DNA (ddPCR presurgery plasma). Percentage reflects fractional abundance of mutant *PIK3CA* (E545K or H1047R) to total *PIK3CA* DNA.

In total, from the 15 *PIK3CA* mutations identified in primary tumors by ddPCR, 14 *PIK3CA* mutations were detected in 13 of the presurgery plasma samples, as 1 patient (patient 4) had concurrent E545K and H1047R mutations identified in both tumor and ptDNA. Of these *PIK3CA*-positive ptDNA samples, 8 patients, including patient 4, had stage IA disease with mutant *PIK3CA* ptDNA fractional abundance ranging from 0.01% to 0.12%. Three patients with stage IIA disease had plasma *PIK3CA* mutation levels with a fractional abundance of 0.02% and 0.01%, whereas 2 stage IIB patients' plasma samples demonstrated mutant *PIK3CA* at 0.01% and 2.99% fractional abundance. Fractional abundance for all ddPCR results are shown in Tables 2 and 3 with 95% CIs in Supplementary Table S4. Representative ddPCR presurgery plasma DNA analyses are shown in Supplementary Fig. S1.

PIK3CA mutations detected in ptDNA by ddPCR after surgery

We then sought to determine whether any patients continued to have detectable mutant *PIK3CA* after breast surgery. Postsurgical plasma specimens were collected from 17 of the 29 patients, including 9 patients with a positive mutant *PIK3CA* presurgical ptDNA sample, and a 10th patient (patient 4) whose tumor and presurgical ptDNA harbored both E545K and H1047R *PIK3CA* mutations for a total of 10 potentially informative samples with 11 mutations identified in presurgery ptDNA. We collected postsurgery blood samples within 14 days after surgery in 5 of these patients as initially planned, whereas logistical issues allowed us to collect the remaining 5 patients' postsurgery blood at times ranging from 15 to 72 days after surgery. Two patients, patients 16 and 22, had their blood collected while

Table 3. ddPCR analysis of postsurgery plasma for patients with presurgery *PIK3CA* mutation-positive ptDNA

Patient	ddPCR presurgery plasma (day 0)	Surgery (type, day)	ddPCR postsurgery plasma (day)	Chemotherapy (day)	Radiation therapy (day)	Endocrine therapy (day)	Clinical information as of 5/2013
1	H1047R (0.03%)	Mastectomy Day 24	H1047R (0.06%) Day 72	None	None	Day >72	No recurrence
4	E545K (0.01%) H1047R (0.02%)	Lumpectomy Day 9	Wild Type H1047R (0.03%) Day 22	None	None	Day 45	No recurrence
5	H1047R (0.02%)	Lumpectomy Day 23	Wild type Day 36	Day >36	Day >36	None	No recurrence
8	H1047R (0.12%)	Mastectomy Day 31	Wild type Day 43	None	None	Day >43	No recurrence
9	E545K (0.02%)	Mastectomy Day 38	Not collected	None	None	Day 77	No recurrence
11	H1047R (0.01%)	Mastectomy Day 37	Not collected	Day 101	None	Day 186	No recurrence
16	H1047R (2.99%)	Mastectomy Day 40	H1047R (0.02%) Day 85	Day 65	Day 261	None	Metastatic disease developed in brain, liver and lung at 26 months from initial diagnosis
17	E545K (0.07%)	Lumpectomy Day 28	Wild type Day 43	Day >78	Day >78	Day >78	No recurrence
19	E545K (0.01%)	Lumpectomy Day 25	Not collected	None	Day 92	Day 128	No recurrence
22	H1047R (0.02%)	Lumpectomy Day 13	Wild type Day 85	None	Day 50	Day 85	No recurrence
25	H1047R (0.01%)	Lumpectomy Day 4	H1047R (0.02%) Day 12	None	Day 71	Day 118	No recurrence
28	H1047R (0.02%)	Lumpectomy Day 29	H1047R (0.01%) Day 42	Day >48	Day >48	Day >48	No recurrence
29	H1047R (0.01%)	Lumpectomy Day 25	Wild type Day 45	Day >45	Day >45	Day >45	No recurrence

NOTE: ddPCR was performed on corresponding plasma DNA prepared from blood collected at various times postsurgery. Percentage reflects fractional abundance of mutant *PIK3CA* (E545K or H1047R) to total *PIK3CA* DNA. For patients who received adjuvant radiation and/or systemic therapies with their local oncologists, the exact day of initiating those therapies is unknown though clinical follow up at our institution documents the minimal time (e.g., Day > XX).

receiving adjuvant chemotherapy and radiation therapy, respectively (Table 3).

Of the 10 patients with *PIK3CA* mutations detected in ptDNA before surgery, 5 patients had detectable ptDNA postsurgery. For patient 1, there was a lengthy period between surgery and the postsurgery blood specimen (48 days), although adjuvant endocrine therapy was initiated after this sample was taken. Interestingly, patient 4 had both *PIK3CA* E545K and H1047R mutations present in tumor and presurgery plasma, but only the H1047R mutation was detected in the postsurgery sample taken 13 days after surgery and before adjuvant endocrine therapy. Thus far, patient 16 is the only patient with a *PIK3CA* mutation who has had a cancer recurrence (26 months after initial diagnosis). She presented with a triple negative (ER/PR/HER2 negative) metaplastic breast cancer, received standard adjuvant chemotherapy and radiation, and had the highest fractional abundance of presurgery mutant *PIK3CA* ptDNA (2.99%). Her postsurgery blood obtained after cycle 2 of adjuvant chemotherapy had persistent albeit lower fractional abundance of mutant *PIK3CA* ptDNA. Patients 25 and 28 had a relatively smaller fractional abundance of mutant *PIK3CA* ptDNA that was similar before and after surgery (lumpectomy for both) with a relatively short period of time between surgery and postsurgery blood draw at days 8 and 13, respectively. Both patients' postsurgery blood samples were collected before adjuvant systemic and radiation therapies.

Discussion

We used the new technique of ddPCR in order to identify *PIK3CA* mutations in FFPE primary tumor samples and corresponding plasma from patients with early-stage breast cancer. We detected *PIK3CA* mutations in presurgery blood samples with 93.3% sensitivity (14/15 mutations) and 100% specificity. In addition, postsurgery blood samples were available for 10 mutation-positive patients and ptDNA was detected in 5 of these patients. Importantly, these patients had no clinical evidence of disease after surgery. This high level of sensitivity and specificity for cancer mutation detection in peripheral blood offers a number of potential clinical applications for oncology practice. First, ddPCR could augment or replace mutation detection in primary cancer tissues. The fact that ddPCR can detect additional mutations in primary tumor tissues not found by traditional Sanger sequencing could increase the number of patients who are candidates for targeted therapies. Interestingly, patient 4′s sample was detected by ddPCR with a high fractional abundance (E545K, 28.9%) but was not identified by sequencing, although in theory 28.9% is within the reported sensitivity of Sanger sequencing. Repeat sequencing of this sample was negative for mutation. Although we do not have a definitive explanation for this, in our hands the use of FFPE DNA for PCR and Sanger sequencing often leads to "allelic drop out" because of degradation of the sample, which could explain these results. In contrast, ddPCR requires much smaller amounts of input DNA and a smaller amplicon size, and therefore is less prone to this phenomenon. Second, the improved sensitivity of ddPCR allows for detection of ptDNA with even low levels of tumor burden. Eight of our patients in whom a *PIK3CA* mutation was detected in presurgery ptDNA had T1 ($\leq$2.0 cm) tumors and node negative disease, with one of these patients harboring a T1b (<1.0 cm) tumor. This presents the opportunity of using blood as a liquid biopsy for mutation detection in patients with early-stage breast cancer without the need for accessing tumor tissues. Third, the short half-life of ptDNA and wide dynamic range may allow for a rapid assessment of therapeutic response for both early- and late-stage disease. Finally, the use of ptDNA detection for minimal residual disease may help guide individualized decisions about adjuvant systemic therapies, and be used for surveillance of patients with a high risk for cancer recurrence.

It should be reemphasized that this is a feasibility study with a relatively small sample size. Thus, the clinical relevance of detectable versus undetectable ptDNA postsurgery is currently unclear. However, it is tempting to speculate that ptDNA may be a useful marker of residual micrometastatic disease and response to adjuvant therapies. The fact that half of our patients with detectable ptDNA before surgery also had ptDNA present after surgery demonstrates that it is feasible to design future trials with longer term follow up to test this hypothesis. Notably, patient 16 with triple negative metaplastic disease had a short disease-free interval despite adjuvant chemotherapy, consistent with the known natural history of this phenotype (30). In contrast, ER/PR–positive breast cancers can recur decades after the diagnosis of early-stage disease, and the use of adjuvant endocrine therapy may play a role in inducing tumor dormancy and increased risk of late recurrences beyond 5 years. Although this would require many years of follow up to address conclusively, such studies could potentially identify patients who might benefit from prolonged or lifelong endocrine therapy, an emerging issue given recent data supporting extended use of these agents in some patients (31, 32).

One of our patients had 2 detectable mutations in her primary tumor as detected by ddPCR. Because there is no biologically known reason why a cancer would have 2 oncogenic mutations in the same gene within a given cell, we would presume that this breast cancer was multiclonal arising from independent progenitors. Given the high-risk nature of some patients for developing breast cancer, this explanation is plausible and in fact has also been reported by us and others in prior studies (5, 33).

Our study provides a proof of concept and attests to the feasibility of ptDNA analysis for patients with early-stage breast cancer; however, there are limitations of our study, including the small sample size and low number of recurrences partly because of the natural history of ER/PR–positive breast cancers. In addition, we assayed only for hotspot mutations in the *PIK3CA* gene and thus were unable to follow approximately half of the patients enrolled in this study. We are currently expanding this study to include multiple blood draws before, during, and after adjuvant therapies and performing broader mutational analyses to obtain additional "markers" to measure patients' ptDNA.

Although the sensitivity and specificity of ddPCR for detecting ptDNA is a technologic achievement, applying these assays for routine use in clinical oncology requires further prospective studies. However, the ability to detect ptDNA before and after surgery lends credence to the idea that assessment of ptDNA may allow for more informed individualized decisions about adjuvant systemic therapies. This could potentially change the current paradigm of overtreatment for patients with early-stage breast cancer.

Disclosure of Potential Conflicts of Interest

B.H. Park is a consultant for GlaxoSmithKline and Novartis; a scientific advisory board member for Horizon Discovery, Loxo Oncology, and LTD; and, under separate licensing agreements between Horizon Discovery, LTD, and The Johns Hopkins University, is entitled to a share of royalties received by the University on the sale of products. The terms of this arrangement are managed by the Johns Hopkins University in accordance with its conflict of interest policies. M.L. Samuels is an employee of RainDance Technologies. D. Maar is an employee of Bio-Rad. No potential conflicts of interest were reported by the other authors.

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