**PIK3CA** mutations in plasma circulating tumor DNA predict survival and treatment outcomes in patients with advanced cancers

E. E. Dumbrava1, S. G. Call1, H. J. Huang1, A. L. Stuckett1, K. Madwani2, A. Adat3, D. S. Hong4, S. A. Piha-Paul1, V. Subbiah5, D. D. Karp1, S. Fu1, A. Naing1, A. M. Tsimberidou1, S. L. Moulder2, K. H. Koenig2, C. H. Barcenas3, B. K. Kee3, D. R. Fogelman1, E. S. Kopetz3, F. Meric-Bernstam4,5 & F. Janku1*

Departments of 1Investigational Cancer Therapeutics (Phase I Clinical Trials Program), Division of Cancer Medicine; 2Breast Medical Oncology; 3Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston; 4Sheikh Khalifa Bin Zayed Al Nahyan Institute for Personalized Cancer Therapy, The University of Texas MD Anderson Cancer Center, Houston, USA; 5Department of Breast Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, USA

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**Background:** Oncogenic mutations in **PIK3CA** are prevalent in diverse cancers and can be targeted with inhibitors of the phosphoinositide-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway. Analysis of circulating tumor DNA (ctDNA) provides a minimally invasive approach to detect clinically actionable **PIK3CA** mutations.

**Patients and methods:** We analyzed **PIK3CA** hotspot mutation frequency by droplet digital PCR (QX 200; BioRad) using 16 ng of unamplified plasma-derived cell-free DNA from 68 patients with advanced solid tumors (breast cancer, n = 41; colorectal cancer, n = 13; other tumor types, n = 14). Results quantified as variant allele frequencies (VAFs) were compared with previous testing of archival tumor tissue and with patient outcomes.

**Results:** Of 68 patients, 58 (85%) had **PIK3CA** mutations in tumor tissue and 43 (74%) **PIK3CA** mutations in ctDNA with an overall concordance of 72% (49/68, k = 0.38). In a subset analysis, which excluded samples from 26 patients known not to have disease progression at the time of sample collection, we found an overall concordance of 91% (38/42, k = 0.74). **PIK3CA**-mutated ctDNA VAF of ≥8.5% (5% trimmed mean) showed a longer median survival compared with patients with a higher VAF (15.9 versus 9.4 months; 95% confidence interval 6.7-17.1 months; P = 0.014). Longitudinal analysis of ctDNA in 18 patients with serial plasma collections (range 2-22 time points, median 5) showed that those with a decrease in **PIK3CA** VAF had a longer time to treatment failure (TTF) compared with patients with an increase or no change (10.7 versus 2.6 months; P = 0.048).

**Conclusions:** Detection of **PIK3CA** mutations in ctDNA is concordant with testing of archival tumor tissue. Low quantity of **PIK3CA**-mutant ctDNA is associated with longer survival and a decrease in **PIK3CA**-mutant ctDNA on therapy is associated with longer TTF.

**Key words:** PIK3CA, circulating tumor DNA, droplet digital PCR, cancer

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**INTRODUCTION**

Aberrant activation of the phosphoinositide-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway can lead to decreased apoptosis and increased cellular proliferation, contributing to cancer progression and therapeutic resistance.1-5 Activation of the PI3K/AKT/mTOR pathway can occur through a variety of mechanisms, including mutations in the p110α (**PIK3CA**) catalytic subunit of PI3K.6,7 Somatic oncogenic mutations in **PIK3CA** commonly occur across varying cancers including gynecologic (uterine, cervical, ovarian, vaginal), breast, colorectal, and non-small-cell lung cancers.8-12 Common ‘hot spots’ of **PIK3CA** somatic mutations that lead to gain of function and activation of the PI3K/AKT/mTOR pathway occur in exon 9 (helical domain, p.E542K and p.E545K) and in exon 20 (kinase domain, p.H1047R or p.H1047L).6,12 **PIK3CA** mutations have been identified as primary oncogenic drivers as well as emerging alterations associated with therapeutic resistance.13-15

**PIK3CA** mutations in cancer can be associated with a favorable response to treatment with PI3K/mTOR pathway inhibitors.10,11,13,16-18 Direct targeting of **PIK3CA** with alpelisib in patients with **PIK3CA**-mutated advanced cancers can have anticancer efficacy, and alpelisib in combination with the selective estrogen receptor degrader, fulvestrant, has been approved for the treatment of patients with hormone...
receptor-positive, PIK3CA-mutated [in tumor and/or circulating tumor DNA (ctDNA)] metastatic breast cancer who were previously treated with hormone therapy. Testing of ctDNA provides a minimally invasive alternative to molecular testing of tumor tissue; however, concordance and sensitivity can differ among technologies and tumor types. We hypothesized that PIK3CA mutations can be detected in a small volume of plasma ctDNA from patients with progressing advanced cancers by droplet digital polymerase chain reaction (ddPCR) and that quantity and dynamic changes in ctDNA assessed by variant allele frequency (VAF) of PIK3CA-mutated ctDNA can correspond with patients’ outcomes.

METHODS

Patients

Patients with advanced or metastatic solid tumors and available PIK3CA mutation status from tumor tissue receiving their care at the University of Texas MD Anderson Cancer Center, who were considered for experimental therapies between November 2010 and December 2017, were enrolled in this study in accordance with MD Anderson’s Institutional Review Board guidelines. Patients were offered to have an optional blood collection prior to and sequentially during their experimental cancer treatment. Patients signed a written informed consent, and this study was conducted in accordance with the Declaration of Helsinki.

Tumor samples were collected during standard-of-care therapeutic and/or diagnostic procedures and genomic testing was performed on archival formalin-fixed paraffin-embedded (FFPE) tumor samples in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory for identification of actionable alterations for personalized cancer therapy. The tumor genomic testing was done by PCR or next-generation sequencing (NGS) on approved targeted gene panels. Clinical characteristics were collected from electronic medical records and prospectively maintained institutional databases.

Plasma-derived circulating tumor DNA analysis

Blood samples were collected into ethylenediaminetetraacetic acid tubes prior to experimental cancer therapy and during therapy whenever possible. Plasma was obtained within 2 h of blood collection by double centrifugation of blood samples. Cell-free DNA was isolated and extracted using the QIAamp circulating nucleic acid kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions.

Using a multiplex ddPCR device (QX 200; BioRad, Hercules, CA), 16 ng of unamplified ctDNA was tested for PIK3CA mutations (p.H1047R, p.H1047L, p.E542K, and p.E545K) versus wild-type alleles, according to the manufacturer’s instructions. For patients with mutations present in the tumor analysis, but not in the plasma ctDNA, re-testing with increased amount of ctDNA (21-247 ng) was performed. The lower limit of detection is <0.1% VAF per single well for the mutation-specific assays. Patients with simultaneous KRAS mutations in tumor tissue were also tested whenever feasible for the presence of corresponding KRAS mutation in ctDNA as described previously.

Treatment and evaluation

Whenever possible, patients with confirmed PIK3CA mutations by FFPE tumor tissue analysis were enrolled on genome-matched clinical trials involving inhibitors of the PI3K/ATK/mTOR pathway. For these patients, treatment was administered in accordance with IRB-approved protocols, and patients received therapy until clinical or radiological disease progression per RECIST version 1.1 or until the development of excessive intolerable treatment-related toxicity. We evaluated the type of targeted therapy that they received and the best overall response on treatment.

Statistical analysis

Concordance between mutation analysis of tumor tissue and ctDNA was calculated using the kappa coefficient. Overall survival (OS) was defined as the time from the first blood collection for ctDNA analysis to the date of death or last follow-up. Time to treatment failure (TTF) was defined as the time from the date of systemic experimental therapy initiation to the date the patient was taken off the treatment or last follow-up. Last follow-up or date of death was determined based on the electronic medical records. The Kaplan–Meier method was used to estimate OS and TTF, and a log-rank test was used to compare OS and TTF among patient subgroups. Multivariate Cox proportional hazards regression was used to assess the prognostic impact of PIK3CA VAF, in addition to other clinical variables including cancer type, blood serum albumin levels, and the presence of simultaneous mutations in the KRAS oncogene.

All tests were two-sided, and P values <0.05 were considered statistically significant. All statistical analyses were performed with SPSS version 23 (SPSS, Chicago, IL) or Prism 7 (GraphPad, San Diego, CA) software program.

RESULTS

Patient characteristics

A total of 68 patients with advanced cancers were analyzed in this study, the majority of which were female (81%), with a median age of 57 years (range 32-82 years). The most common cancer diagnosis among these patients was breast cancer (n = 41, 60%), followed by colorectal cancer (n = 13, 19%), non-small-cell lung cancer (n = 3, 4%), ovarian cancer (n = 3, 4%), salivary gland cancer (n = 2, 3%), and other cancers (n = 6, 9%). There was a median of 27 months (0-189 months) between the archival tumor tissue collection and plasma collections. Molecular testing of archival tumor tissue was carried out using either PCR (n = 12, 18%) or NGS (n = 56, 82%) as a part of routine clinical care. Based on tumor tissue molecular testing, 10 patients (15%) had
**PIK3CA wild-type tumors, while the remaining patients had the following PIK3CA mutations: E542K (n = 10, 15%), E545K (n = 17, 23%), H1047L (n = 5, 7%), and H1047R (n = 29, 40%). Patient characteristics are summarized in Table 1.**

**PIK3CA mutation concordance between circulating tumor DNA and tumor tissue**

Among the 68 patients included in this study, 10 (15%) had wild-type PIK3CA and 58 (85%) had PIK3CA mutations in the tumor tissue. Analysis of plasma ctDNA samples collected before starting therapy detected PIK3CA mutations in 39 samples with known mutations in the tumor tissue, resulting in observed agreement rate of 72% [49/68; \( \kappa = 0.38 \), standard error (SE) = 0.09; 95% confidence interval (CI) 0.19-0.56], sensitivity of 67% [39/68; 95% CI 0.54-0.79], and specificity of 100% (10/10; 95% CI 0.69-1; Table 2). We hypothesized that using a higher input of DNA can improve the sensitivity of ddPCR. Of 19 false-negative plasma samples, we re-tested nine samples with available residual material using a higher input of DNA (range 22-227 ng) and detected PIK3CA mutations in four additional samples, improving overall agreement to 78% [53/68; \( \kappa = 0.46 \), SE = 0.10; 95% CI 0.25-0.66] and sensitivity to 74% [43/68; 95% CI 0.61-0.85]. Finally, we evaluated concordance by excluding samples from 26 patients known not to have disease progression at the time of sample collection to account for samples that might have undetectable levels of plasma ctDNA due as result of therapy, and found an overall agreement of 91% (38/42; \( \kappa = 0.74 \), SE = 0.12; 95% CI 0.51-0.97) and sensitivity of 88% (30/42; 95% CI 0.73-0.97; Table 2).

**PIK3CA mutations in circulating tumor DNA and survival**

We next analyzed the association between the quantity (determined by VAF) of PIK3CA-mutant ctDNA and patients’ OS. To eliminate potential bias from samples with no detectable PIK3CA-mutated ctDNA, we divided patients into two groups using a 5% trimmed mean instead of the median, which was 8.5% VAF. Patients with VAF PIK3CA-mutated ctDNA \( \leq 8.5% \) showed longer median OS (15.9 months; 95% CI 10.6-21.2 months) compared with patients with VAF >8.5% (9.4 months; 95% CI 3.8-15.0 months; \( P = 0.014 \); Figure 1A). In addition, we analyzed the association between OS and additional clinical factors such as cancer type, serum albumin levels, and the presence of simultaneous ctDNA KRAS mutations and found that patients with breast cancer had longer OS (22.8 months; 95% CI 16.9-28.7 months) compared with patients with other cancers (8.0 months; 95% CI 4.1-11.9 months; \( P = 4 \times 10^{-6} \); Figure 1B); patients with blood serum albumin levels \( \geq 3.5 \) g/ml had longer OS (15.4 months; 95% CI 9.8-21.0 months) compared with patients with albumin levels <3.5 g/ml (4.2 months; 95% CI 0.8-7.6 months; \( P = 0.003 \); Figure 1C); and finally patients with simultaneous ctDNA KRAS mutations had shorter OS (9.9 months; 95% CI 3.1-16.7 months) compared with patients with ctDNA wild-type KRAS (15.7 months; 95% CI 9.0-22.4 months; \( P = 0.022 \); Figure 1D).

A covariate Cox proportional hazards regression model, which included quantity of PIK3CA-mutated ctDNA (VAF \( \leq 8.5% \) versus >8.5%), tumor type (breast versus other cancers), albumin levels (\( \geq 3.5 \) g/ml versus <3.5 g/ml), and simultaneous KRAS mutations in ctDNA (absent versus present), showed that PIK3CA-mutated VAF \( \leq 8.5% \) [hazard ratio (HR) 0.394; 95% CI 0.203-0.766; \( P = 0.006 \)], breast cancer (HR 0.276; 95% CI 0.144-0.529; \( P = 1 \times 10^{-4} \)), and blood serum albumin levels \( \geq 3.5 \) g/ml (HR 0.129; 95% CI 0.035-0.467; \( P = 0.002 \)) were independent prognostic indicators for longer OS (Table 3).

**Longitudinal assessment of PIK3CA mutations in circulating tumor DNA**

Serial plasma ctDNA samples of two or more collections (median 5; range 2-22) before and during administration of systemic therapy were available for 18 patients (2 collections, \( n = 6 \); 3 collections, \( n = 3 \); 4 collections, \( n = 1 \); 6 collections, \( n = 2 \); 7 collections, \( n = 1 \); 8 collections, \( n = 3 \); 9 collections, \( n = 1 \); and 22 collections, \( n = 1 \)). Among these 18 patients, 10 (56%) showed either a decrease or no change in quantity of PIK3CA-mutated ctDNA VAF at the time of the first follow-up, while the remaining eight (44%) showed an increase. Patients with a decrease or no change in PIK3CA VAF had longer median TTF compared with patients showing an increase (155 days versus 84 days; HR 0.42; 95% CI 0.14-1.21; \( P = 0.048 \); Figure 2A).

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**Table 1. Patients characteristics**

| Characteristic | All patients (\( N = 68 \)) |
|---------------|-----------------------------|
| Age at diagnosis - range, years | 32-82 |
| Median | 57 |
| Mean ± standard deviation | 57 ± 12 |
| Gender, n (%) | Female 55 (81), Male 13 (19) |
| Cancer type, n (%) | Breast cancer 41 (60), Colorectal cancer 13 (19), Non-small-cell lung cancer 3 (4), Ovarian cancer 3 (4), Salivary gland cancer 2 (3), Appendiceal cancer 1 (1), Cervical cancer 1 (1), Endometrial cancer 1 (1), Head and neck squamous cell cancer 1 (1), Neuroendocrine cancer 1 (1), Thyroid cancer 1 (1) |
| Type of tumor tissue testing, n (%) | Polymerase chain reaction 12 (18), Next-generation sequencing 56 (82) |
| PIK3CA mutation status in tumor, n (%) | E542K 10 (15), E545K 17 (23), H1047L 5 (7), H1047R 29 (40), Wild-type 10 (15) |
| Time between tumor tissue and plasma collection, months (range) | 27 (0-189) |
In many patients with serial plasma collections, the dynamic changes in VAF of *PIK3CA*-mutated ctDNA dynamically tracked with cancer clinical course. For example, in a 62-year-old female with metastatic metaplastic breast cancer with a *PIK3CA*^{E545K} mutation treated with a combination of liposomal doxorubicin, bevacizumab, and everolimus, we observed significant differences in OS based on differing VAF levels. Patients with a PIK3CA VAF ≥ 8.5% showed a median OS of 15.9 months compared with 9.4 months for patients with a VAF > 8.5% (*P* = 0.014). Patients with breast cancer had a median OS of 22.8 months compared with 8.0 months for patients with other cancers (*P* = 4 × 10⁻⁶). Patients with serum albumin levels ≥ 3.5 g/ml had a median OS of 15.4 months compared with 4.2 months for patients with albumin levels < 3.5 g/ml (*P* = 0.003). Patients with mutations in the KRAS oncogene had a shorter median OS of 9.9 months compared with 15.7 months for patients lacking mutations in KRAS (*P* = 0.022).

### Table 2. Concordance between PIK3CA mutation testing in the tumor and ctDNA

| DNA Input        | PIK3CA Mutation in Both FFPE and ctDNA | PIK3CA Wild-type in Both FFPE and ctDNA | PIK3CA Mutation in FFPE Only | PIK3CA Mutation in ctDNA Only | Observed Agreements | Sensitivity (95% CI) | Specificity (95% CI) |
|------------------|----------------------------------------|-----------------------------------------|-------------------------------|-------------------------------|----------------------|----------------------|----------------------|
| 16 ng DNA input  | 39                                     | 10                                      | 19                            | 0                             | 49 (72%); *κ* = 0.38, SE = 0.09; 95% CI 0.19-0.56 | 67% (0.54-0.79)      | 100% (0.69-1)        |
| Up to 227 ng DNA input (9 samples retested) | 43                                     | 10                                      | 15                            | 0                             | 53 (78%); *κ* = 0.46, SE = 0.10; 95% CI 0.25-0.66 | 74% (0.61-0.85)      | 100% (0.69-1)        |
| Up to 227 ng DNA (excluding patients without disease progression at time of sample collection) | 30                                     | 8                                       | 4                             | 0                             | 38 (91%); *κ* = 0.74, SE = 0.12; 95% CI 0.51-0.97 | 88% (0.73-0.97)      | 100% (0.63-1)        |

CI, confidence interval; ctDNA, circulating tumor DNA; FFPE, formalin-fixed paraffin-embedded; SE, standard error.

Figure 1. Kaplan-Meier survival of PIK3CA variant allele frequency (VAF) and other clinical variables. Kaplan-Meier survival calculations were performed to assess the impact of clinical variables on patient overall survival (OS). (A) Patients with a PIK3CA VAF ≤ 8.5% showed significantly longer median OS compared with patients with a VAF > 8.5% (15.9 months compared with 9.4; *P* = 0.014). (B) Patients diagnosed with breast cancer had longer OS compared with patients with other cancers (22.8 months compared with 8.0; *P* = 4 × 10⁻⁶). (C) Patients with serum albumin levels ≥ 3.5 g/ml had longer OS compared with patients with other cancers (15.4 months compared with 4.2; *P* = 0.003). (D) Patients with mutations in the KRAS oncogene had shorter OS compared with patients lacking mutations in KRAS (9.9 months compared with 15.7; *P* = 0.022).
initially observed a decrease in VAF of PIK3CA-mutated ctDNA followed by a steady rise from cycle 3 day 1 until radiological progression on computed tomography (CT) because of new liver metastases, which resulted in treatment discontinuation after six cycles (Figure 2B). Another example is a 66-year-old female patient with endometrial cancer with PIK3CAH1047L mutation discontinuation after six cycles (Figure 2B). Another example is a 66-year-old female patient with endometrial cancer with PIK3CAH1047L mutation.

**DISCUSSION**

Blood-based detection of PIK3CA mutations is of increasing importance as PIK3CA mutation status can change over time due to clonal evolution and emergence of therapeutic resistance. In addition, patients with hormone receptor-dependent metastatic breast cancer and PIK3CA mutation in ctDNA had longer progression-free survival when the PI3K inhibitor buparlisib was added to hormone therapy with fulvestrant. Our study demonstrated that detection of PIK3CA mutations in blood-derived ctDNA by ddPCR in patients with advanced cancers referred for experimental therapies is feasible and concordant with standard of care testing of tumor tissue, especially in blood collected from patients experiencing disease progression (increase in concordance from 72% to 91% and sensitivity from 67% to 88%). In addition, our method demonstrated high specificity with no false-positive results. We also demonstrated that sensitivity increases with an increase in DNA input, which in our study resulted in conversion of 44% of initially falsely negative ctDNA samples. Our results are similar to previously presented studies. For instance, Higgins et al. reported concordance of 72.5% for plasma PIK3CA mutation detection with BEAMing (beads, emulsion, amplification, magnetics) PCR compared with discordantly tested archival FFPE tissue.

Our study also demonstrated that patients with high VAF of mutant ctDNA in baseline samples had shorter OS compared with patients with low VAF, and this observation has been confirmed on the multivariate analysis ($P = 0.006$). This agrees with previously published data. For instance, our group and others demonstrated shorter OS in patients with high VAF of mutant ctDNA for multiple oncogenic mutations detected by digital PCR or NGS technologies. However, it remains unclear if high VAF simply represents increased tumor burden or rather different and more aggressive biology.

We also noticed that patients with an increase in ctDNA quantity defined by delta in VAF during therapy had shorter TTF than patients with a decrease or no change ($P = 0.048$). This is not unexpected. Although the ctDNA quantity can fluctuate during therapy, there is a mounting evidence that negative delta in ctDNA quantity during therapy is associated with better treatment outcomes. For instance, our group and others reported similar observations for patients with BRAF-mutated cancers, KRAS-mutated cancers, and other cancers treated with diverse systemic cancer therapies. More recently, Pascual et al. reported that changes in quantity of PIK3CA-mutated ctDNA in patients with PIK3CA-mutated tumors treated with the PI3K inhibitor taselisib-based therapy are associated with treatment outcomes, and specifically patients with high on-treatment ctDNA had shorter progression-free survival ($P = 0.04$). We also observed that the dynamic changes in VAF of PIK3CA-mutated ctDNA correlated with clinical course and were perhaps sometimes even more suggestive of clinical outcomes than standard imaging. Systematic studies to develop ctDNA-guided approaches to assess response to systemic therapies are currently underway by our group and others.

Our study also has several limitations. First, the study was retrospective and included a relatively small number of patients with diverse cancer types. Therefore, our observations suggesting clinical utility for assessment of OS and TTF need to be validated in prospective clinical studies with a more homogenous patient population and treatment selection. Second, we tested only for four most frequent PIK3CA mutations in exons 9 and 20. Third, we used archival tumor tissue, which was not collected at the same time as plasma samples and also 26 patients did not have disease progression at the time of plasma sample collection, which could have negatively affected our concordance rates and sensitivity.

In summary, despite the aforementioned limitations, our data suggest that commonly occurring mutations in PIK3CA can be detected by ddPCR in plasma ctDNA with high

| Clinical variable | Hazard ratio | 95% Confidence interval | $P$ value |
|-------------------|--------------|-------------------------|-----------|
| PIK3CA variant allele frequency ($\geq 8.5\%$ versus $>8.5\%$) | 0.394 | 0.203-0.766 | 0.006 |
| Cancer diagnosis (breast versus other) | 0.276 | 0.144-0.529 | 0.0001 |
| Serum albumin ($\geq 3.5$ $g/ml$ versus $<3.5$ $g/ml$) | 0.129 | 0.035-0.467 | 0.002 |
| KRAS comutations (no comutation versus comutation) | 0.851 | 0.393-1.843 | 0.682 |

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sensitivity and specificity in patients with progressing cancer. Low amount of PIK3CA-mutant cfDNA is associated with longer OS. Changes in PIK3CA VAF could be an early surrogate biomarker for TTF on systemic treatments.

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