The Use of Serial Circulating Tumor DNA to Detect Resistance Alterations in Progressive Metastatic Breast Cancer

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ABSTRACT

Purpose: Circulating tumor DNA (ctDNA) is a promising tool for noninvasive longitudinal monitoring of genomic alterations. We analyzed serial ctDNA to characterize genomic evolution in progressive metastatic breast cancer.

Experimental Design: This was a retrospective cohort between 2015 and 2019 obtained under an Institutional Review Board–approved protocol at Northwestern University (Chicago, IL). ctDNA samples were analyzed with Guardant360 next-generation sequencing (NGS) assay. A total of 86 patients had at least two serial ctDNA collections with the second drawn at first post-NGS progression (PN1) by imaging and clinical assessment. A total of 27 participants had ctDNA drawn at second post-NGS clinical progression (PN2). We analyzed alterations, mutant allele frequency (MAF), number of alterations (NOA), and sites of disease on imaging in close proximity to ctDNA evaluation. Matched pairs’ variations in MAF, NOA, and alterations at progression were tested through Wilcoxon test. We identified an independent control cohort at Massachusetts General Hospital (Boston, MA) of 63 patients with serial ctDNA sampling and no evidence of progression.

Results: We identified 44 hormone receptor–positive, 20 HER2+, and 22 triple-negative breast cancer cases. The significant alterations observed between baseline and PN1 were TP53 (P = 0.0075), PIK3CA (P < 0.0126), AR (P < 0.0126), FGR1 (P < 0.0455), and ESR1 (P < 0.0143). Paired analyses revealed increased MAF and NOA from baseline to PN1 (P = 0.0026, and P < 0.0001, respectively). When compared with controls without progression, patients with ctDNA collection at times of progression were associated with increased MAF and NOA (P = 0.0042 and P < 0.0001, respectively).

Conclusions: Serial ctDNA testing identified resistance alterations and increased NOA and MAF were associated with disease progression. Prospective longitudinal ctDNA evaluation could potentially monitor tumor genomic evolution.
However, the presence of mutations in studies comparing tissue NGS and ctDNA in treatment outcomes. In breast cancer, there is a lack of prospective randomized phase III trial of patients with hormone receptor therapy in a prospective clinical trial with neratinib (19). Similarly, a be useful for selection and prediction of beneﬁcious of ctDNA as a tool for monitoring tumor genetic evolution and detecting resistance alterations in the setting of progressive disease.

Tumor genomic evolution in metastatic breast cancer poses a signiﬁcant treatment challenge at times of progression. Circulating tumor DNA (ctDNA) has emerged as a promising, noninvasive tool to detect alterations in peripheral blood over time. However, there are limited data on the use of ctDNA to identify resistance alterations with serial blood sampling. We analyzed serial ctDNA of 86 patients collected at clinical progression across breast cancer subtypes. We observed that increasing mutant allele frequency and number of alterations were associated with disease progression. We identiﬁed several key resistance alterations, namely, TP53, PIK3CA, ESR1, FGFR1, AR, and ERBB2. These ﬁndings highlight the potential of ctDNA as a tool for monitoring tumor genetic evolution and detecting resistance alterations in the setting of progressive disease.

Patients with MBC enrolled in this study were identiﬁed retrospectively within a single institution, Northwestern University (Chicago, IL), between 2015 and 2019. The Institutional Review Board at the Robert H. Lurie Comprehensive Cancer Center (Chicago, IL) approved the study. Given the deidentiﬁed and retrospective nature of the study, requirement for informed consent was waived. All investigations were performed in accordance with the statutes set by the Health Insurance Portability and Accountability Act and the Declaration of Helsinki. We identiﬁed 255 patients with MBC that had ctDNA NGS as part of their standard of care. Cases of MBC were conﬁrmed by patient chart review. Of these 255, 86 patients had more than one ctDNA collection. The ﬁrst ctDNA collection by each patient was identiﬁed and deﬁned as the baseline collection. This baseline ctDNA assessment was performed as part of routine clinical practice, and in many cases, patients had already received several lines of prior therapy. These patients were then followed from the baseline time point until evidence of ﬁrst progression after baseline NGS. This progression was deﬁned as evidence of clinical or radiographic progression resulting in change of treatment. ctDNA at time of progression (within 1 month of treatment change) was analyzed as a marker of genomic evolution at ﬁrst progression. This time point was then deﬁned as ctDNA collection at the ﬁrst progression after baseline ctDNA collection, or P1. A smaller subset of 27 had a third ctDNA analysis taken at second progression following baseline NGS analysis. Thus, ctDNA collected at the second progression after baseline ctDNA collection was designated as P2. While change of treatment occurred within 1 month of ctDNA collection, this was either prior to or after collection. Each chart was reviewed for pertinent clinical factors such as date of progression, cancer subtype, total lines of therapy, and sites of metastatic disease. Subtypes were deﬁned as HR− (HER2−), HER2+ (any HR status), and triple-negative breast cancer (TNBC). Sites of metastatic disease were deﬁned using imaging within 1 month of ctDNA collection. ctDNA was analyzed for type of alteration, NOA, and MAF. NGS analyses performed on tumor tissue specimens were also analyzed and compared with ctDNA at baseline. All clinical data gathered from chart review were stored using a secure, password-protected REDCap database.

An additional control cohort of 62 patients was identiﬁed from a second academic institution (Massachusetts General Hospital, Boston, MA) who also underwent serial ctDNA testing with the same assay (Guardian360) as part of routine clinical practice. This cohort included patients with MBC who underwent a baseline pretreatment ctDNA collection (collected before the start of next line of therapy) as well as an on-treatment ctDNA collection performed during treatment.

Materials and Methods

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Translational Relevance

Tumor genomic evolution in metastatic breast cancer poses a signiﬁcant treatment challenge at times of progression. Circulating tumor DNA (ctDNA) has emerged as a promising, noninvasive tool to detect alterations in peripheral blood over time. However, there are limited data on the use of ctDNA to identify resistance alterations with serial blood sampling. We analyzed serial ctDNA of 86 patients collected at clinical progression across breast cancer subtypes. We observed that increasing mutant allele frequency and number of alterations were associated with disease progression. We identiﬁed several key resistance alterations, namely, TP53, PIK3CA, ESR1, FGFR1, AR, and ERBB2. These ﬁndings highlight the potential of ctDNA as a tool for monitoring tumor genetic evolution and detecting resistance alterations in the setting of progressive disease.

Tumor genomic evolution in metastatic breast cancer poses a signiﬁcant treatment challenge at times of progression. Circulating tumor DNA (ctDNA) has emerged as a promising, noninvasive tool to detect alterations in peripheral blood over time. However, there are limited data on the use of ctDNA to identify resistance alterations with serial blood sampling. We analyzed serial ctDNA of 86 patients collected at clinical progression across breast cancer subtypes. We observed that increasing mutant allele frequency and number of alterations were associated with disease progression. We identiﬁed several key resistance alterations, namely, TP53, PIK3CA, ESR1, FGFR1, AR, and ERBB2. These ﬁndings highlight the potential of ctDNA as a tool for monitoring tumor genetic evolution and detecting resistance alterations in the setting of progressive disease.

Prior studies have identiﬁed circulating tumor cells (CTC) as important markers of disease; however, changing therapy in response to changes in CTC was not associated with improved patient outcomes (21). Similarly, ctDNA has been identiﬁed as a potential marker of overall disease burden. For example, in both breast and lung cancer, decreasing ctDNA content has shown tumor response prior to clinical detection (6, 22). Conversely, increasing levels have shown to be a predictor of progression prior to detect anatomic progression on imaging (23–25). Furthermore, some studies have suggested that ctDNA detection after treatment may be an important predictor of minimal residual disease and cancer recurrence, particularly when serial analyses were performed (26, 27). Increasing number of alterations (NOA) or increasing percent of the dominant mutant allele frequency (MAF) has been associated with worse clinical outcomes (6, 28, 29). MAF has also been shown to be a marker of tumor burden (5, 6). Previous studies have shown that rapid increase in MAF burden correlated with progressive disease and subsequently declined with successful therapy across multiple tumor types (5, 6, 30, 31). In cases where multiple subclones were found, relative changes within each clone have been an important predictor of tumor evolution and tumor response to treatment (32, 33). However, despite the data linking ctDNA and tumor burden, studies examining the use of ctDNA and effects on patient survival in breast cancer are lacking.

ctDNA is already being investigated as a potential method of detecting resistance alterations in breast cancer. This is particularly well studied in the setting of HR− MBC where endocrine therapy has led to the development of clones expressing endocrine resistance, including ESR1 alterations, RB1 alterations, PIK3CA driver alterations, and ERBB2 alterations (34–37). Copy-number variants (CNV) of FGFR1, ERBB2, and CCND1 have also been associated with endocrine resistance (38, 39). However, while data exist describing resistance alterations on HR− disease, there is still a lack of data describing resistance alterations and their actionability across subtypes. Furthermore, information regarding resistance alterations at later time points is needed.

In this study, we sought to characterize the clinical and prognostic impacts of ctDNA monitoring in MBC. More speciﬁcally, we followed patients with progressive MBC who had serial ctDNA samples drawn at times of clinical progression to describe resistance proﬁles. We have outlined overall landscape of resistance alterations, particularly alterations most likely to be seen at the time of clinical progression.

Materials and Methods

Patients with MBC enrolled in this study were identiﬁed retrospectively within a single institution, Northwestern University (Chicago, IL), between 2015 and 2019. The Institutional Review Board at the Robert H. Lurie Comprehensive Cancer Center (Chicago, IL) approved the study. Given the deidentiﬁed and retrospective nature of the study, requirement for informed consent was waived. All investigations were performed in accordance with the statutes set by the Health Insurance Portability and Accountability Act and the Declaration of Helsinki. We identiﬁed 255 patients with MBC that had ctDNA NGS as part of their standard of care. Cases of MBC were conﬁrmed by patient chart review. Of these 255, 86 patients had more than one ctDNA collection. The ﬁrst ctDNA collection by each patient was identiﬁed and deﬁned as the baseline collection. This baseline ctDNA assessment was performed as part of routine clinical practice, and in many cases, patients had already received several lines of prior therapy. These patients were then followed from the baseline time point until evidence of ﬁrst progression after baseline NGS. This progression was deﬁned as evidence of clinical or radiographic progression resulting in change of treatment. ctDNA at time of progression (within 1 month of treatment change) was analyzed as a marker of genomic evolution at ﬁrst progression. This time point was then deﬁned as ctDNA collection at the ﬁrst progression after baseline ctDNA collection, or P1. A smaller subset of 27 had a third ctDNA analysis taken at second progression following baseline NGS analysis. Thus, ctDNA collected at the second progression after baseline ctDNA collection was designated as P2. While change of treatment occurred within 1 month of ctDNA collection, this was either prior to or after collection. Each chart was reviewed for pertinent clinical factors such as date of progression, cancer subtype, total lines of therapy, and sites of metastatic disease. Subtypes were deﬁned as HR− (HER2−), HER2+ (any HR status), and triple-negative breast cancer (TNBC). Sites of metastatic disease were deﬁned using imaging within 1 month of ctDNA collection. ctDNA was analyzed for type of alteration, NOA, and MAF. NGS analyses performed on tumor tissue specimens were also analyzed and compared with ctDNA at baseline. All clinical data gathered from chart review were stored using a secure, password-protected REDCap database.

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Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Baseline</th>
<th>PN1</th>
<th>PN2</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;45</td>
<td>51 (58.6%)</td>
<td>51 (58.6%)</td>
<td>7 (25.9%)</td>
<td>16 (58.0%)</td>
</tr>
<tr>
<td>45–65</td>
<td>17 (19.5%)</td>
<td>17 (19.5%)</td>
<td>7 (25.9%)</td>
<td>16 (58.0%)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>11 (12.6%)</td>
<td>10 (11.5%)</td>
<td>3 (11.1%)</td>
<td>9 (33.3%)</td>
</tr>
</tbody>
</table>

Inflammatory breast cancer
- No alterations: 47 (55.2%) Baseline, 47 (55.2%) PN1, 13 (48.1%) PN2, Not available Controls
- Yes alterations: 38 (44.7%) Baseline, 38 (44.7%) PN1, 14 (51.8%) PN2, Not available Controls

Cancer subtype
- HR\(^+\) (HER2\(^+\)): 44 (50.5%) Baseline, 44 (50.5%) PN1, 14 (51.8%) PN2, 54 (87.1%) Controls
- HER2\(^+\) (any HR): 20 (22.9%) Baseline, 20 (22.9%) PN1, 9 (33.3%) PN2, 2 (5.2%) Controls
- Triple negative: 23 (26.4%) Baseline, 23 (26.4%) PN1, 14 (48.1%) PN2, 6 (9.6%) Controls

Average prior lines of therapy
- HR\(^+\) (HER2\(^+\)): 3 (IQR 1–6) Baseline, 4 (IQR 2–7) PN1, 5 (IQR 3–8) PN2, 3 (IQR 2–4) Controls
- HER2\(^+\) (any HR): 3 (IQR 1–5) Baseline, 4 (IQR 2–6) PN1, 5 (IQR 3–7) PN2, 2 (IQR 1–2) Controls
- Triple negative: 2 (IQR 1–4) Baseline, 3 (IQR 2–5) PN1, 4 (IQR 3–4) PN2, 2 (IQR 1–2) Controls

Metastatic sites
- Visceral only: 45 (51.7%) Baseline, 40 (45.9%) PN1, 11 (40.7%) PN2, 45 (77.9%) Controls
- Nonvisceral: 51 (58.2%) Baseline, 55 (64.1%) PN1, 24 (59.2%) PN2, 22 (22.3%) Controls
- Bone only: 11 (12.6%) Baseline, 10 (11.5%) PN1, 3 (11.1%) PN2, 9 (55.5%) Controls
- Number of alterations: 3.3 (IQR 0.3–11.4) Baseline, 3.6 (IQR 0.8–15.4) PN1, 5.0 (IQR 0.5–14.0) PN2, 3.2 (IQR 1.0–10.1) Controls
- Mutant allele frequency: 0.38 (40.4%) Baseline, 0.33 (27.3%) PN1, 0.26 (28.5%) PN2, Not available Controls
- Pathogenic alterations: 100 (27.6%) Baseline, 100 (27.6%) PN1, 67 (28.5%) PN2, Not available Controls

Note: Shown are clinical and pathologic characteristics of patients with progressive metastatic breast cancer who underwent serial ctDNA analysis at baseline and two additional time points. These time points are the first progression postbaseline NGS (PN1) and the second progression postbaseline NGS (PN2). Shown here also are characteristics of a control group of patients with serial ctDNA who did not show signs of progression (nonprogressor controls).
group received anti-HER2+ therapy with the most common treatment being trastuzumab. In the TNBC group, most patients received chemotherapy.

Associations between baseline MAF, NOA, and survival

Associations between baseline MAF and NOA are depicted in Supplementary Fig. S1. For this analysis, patients were divided according to median MAF (<3.3% or ≥3.3%) and NOA (<4 or ≥4). We found that patients under the median MAF had an improved PFS compared with those above the median MAF (P = 0.0135) but observed no difference in overall survival (OS; P = 0.1230). Patients with NOA <4 experienced longer PFS (P = 0.0114) and OS (P = 0.0380) compared with patients with NOA ≥4.

Changes in MAF across time points

MAF was analyzed at each progression as percent change from baseline (Fig. 1). The highest MAF detected in each case was selected as the representative MAF. When analyzed for the alteration with the NOA fold-variation, P = 0.0444. Baseline PN1

Figure 1.

Patients-wise (A and B, and G–J) and population-wise (C–F) changes to MAF and NOA by time point. Changes to MAF and NOA are reported as fold-variation in the cohort of patients with progressive disease (PD; A, B, I, and J) and patients without progressive disease (No PD; G and H). P values were calculated using the Wilcoxon signed-rank test to assess paired changes between time points. The two cohorts were also compared using boxplots. MAF and NOA were tested at baseline assessment for patients with and without disease progression (C and E) and at second time point (D and F). P values were calculated using the Mann–Whitney U test.
highest MAF, median MAF for the HR+ group was 3.33% (IQR: 0.3–11.4), HER2+ group was 3.55% (IQR: 0.8–15.4), and TNBC group was 4.95% (IQR: 0.5–14). No statistical differences were observed in MAF between groups. Six patients at PN1 and 5 patients at PN2 were not included in analyses due to amplifications in detected alterations rather than mutations. Between baseline and PN1, analysis of paired samples showed 47 cases had an increase of MAF, 27 cases had a decrease in MAF, and six cases had no change. Between baseline to PN1, there was a statistically significant average increase of 132% (P = 0.0026). Between PN1 and PN2, 15 cases had an increase, seven cases had a decrease, and one case had no change in MAF. There was an average increase of 300%. However, this was not statistically significant (P = 0.2768).

Changes in NOA across time points

NOA was analyzed as percent change from baseline (Fig. 1). Median NOA in HR+ was 4 (IQR: 1–6), HER2+ was 5 (IQR: 3–8), and TNBC was 6 (IQR: 4–10). Between baseline and PN1, analysis of paired samples showed 50 cases with an increase, 18 cases with a decrease in NOA, and 18 cases with no change. There was a mean increase in NOA of 128% (P < 0.0001). Between PN1 and PN2, 22 cases had an increase of NOA, 2 had a decrease, and 3 had no change. There was an overall increase of about 212% (P = 0.0022).

Changes to MAF and NOA compared with the control cohort

A comparison of MAF and NOA between the two cohorts is depicted in Fig. 1. When compared with the control cohort, we observed no difference in MAF at baseline collection (P = 0.5963; Fig. 1C). However, when comparing PN1 with the first post-NGS collection for “nonprogressors,” we observed a significantly higher MAF for patients who progressed (P = 0.0042; Fig. 1D). When changes in MAF were investigated across single patients, a significant decrease was observed in patients who did not progress (P = 0.0436; Fig. 1G).

When compared with the control cohort, we observed no difference in NOA at baseline collection (P = 0.9832; Fig. 1E). However, when comparing PN1 with the first post-NGS collection for “nonprogressors,” we observed a significantly larger NOA for patients who progressed (P < 0.0001; Fig. 1F). Paired changes for NOA showed a significant decrease in patients who did not progress (P = 0.0444; Fig. 1H).

Oncogenicity of detected alterations

Using the OncoKB database, oncogenicity of alterations at each time point was assessed (Table 1). On the basis of existing clinical and preclinical data, alterations were categorized as either “oncogenic” or “likely oncogenic.” At baseline, 139 alterations (38.4%) were considered “oncogenic” and 100 alterations (27.6%) were considered “likely oncogenic.” At PN1, there were 166 (33.27%) “oncogenic” alterations and 139 (27.26%) “likely oncogenic” alterations. At PN2 there were 63 (26.81%) “oncogenic” alterations and 67 (28.51%) “likely oncogenic” alterations.

Landscape of alterations across time points

Alterations detected at each time point are depicted in Fig. 2 along with MAF and a heatmap representing absolute number of alterations. These analyses took into account both mutations (SNVs, insertions, deletions) and amplifications. The most common alterations at all three time points were within the TP53 gene. Other commonly detected alterations were in the genes PIK3CA, ESR1, and ERBB2. Across all three time points, PIK3CA was associated with the highest MAF though decreasing at each successive time point. At baseline and PN1, TP53 was associated with the second highest MAF. Most alterations were detected as SNVs with CNV the next most commonly observed alteration. Individual analyses of CNVs or mutations such as SNV, insertions and deletions of each gene showed no statistical significance, likely due to smaller sample size.

Emergence of possible resistance alterations

Across time points, the biggest increase in NOA was observed with TP53. Between baseline and PN1, PIK3CA demonstrated the next highest increase followed by ESR1, FGFR1, and ARID1A (Fig. 3A). Between PN1 and PN2, next to TP53, ESR1 had the largest increase in NOA followed by ERBB2, PDGFR, and MYC. PIK3CA had no change between PN1 and PN2. Across subgroups PN1 was significantly associated with increased NOA of the alterations TP53 (P < 0.0075), PIK3CA (P < 0.0126), ESR1 (P = 0.0143), AR (P < 0.0126), and FGFR1 (P < 0.0453). Changes in NOA of TP53, PIK3CA, ESR1, AR, and FGFR1 between each time point is depicted in Fig. 3B and C and E–G, respectively. Given the frequency of detected ERBB2 alterations within our cohort as well as potential for clinical actionability, changes in NOA for ERBB2 are also depicted in Fig. 3D.

Of the two most commonly detected gene alterations, TP53 and PIK3CA, we observed a mean increase in NOA between baseline and PN1 (0.225 and 0.1625, respectively). TP53 mean NOA also increased between PN1 and PN2 (0.346) while PIK3CA showed no mean change. Paired analysis showed changes in TP53 to be significant between both time points (P = 0.0101 and P = 0.0329) and PIK3CA to be significant between baseline and PN1 (P = 0.0043). The subgroup of patients with TP53 alterations included 19 HR+, 8 HER2+, and 18 TNBC. The subgroup of patients with PIK3CA alterations included 19 HR+, 6 HER2+, and 6 TNBC.

Other alterations of note were ESR1 and AR which both showed mean increases between baseline and PN1 with statistically significant changes in paired analyses (P = 0.0360 and P = 0.0455, respectively). ESR1 also had a statistically significant increase in paired analysis from PN1 to PN2 (P = 0.0255) while AR did not. The subgroup of patients with ESR1 alterations included 12 HR+, 0 HER2+, and 0 TNBC. The subgroup of patients with AR alterations included 9 HR+, 0 HER2+, and 5 TNBC.

Changes to NOA of ERBB2 and FGFR1 were not significant at either time point when paired analysis was performed regardless of subtype. The subgroup of patients with ERBB2 alterations included 5 HR+, 9 HER2+, and 2 TNBC. The subgroup of patients with FGFR1 alterations included 4 HR+, 4 HER2+, and 0 TNBC. ERBB2 was associated with significant change between PN1 and PN2 (P = 0.0474) when analyzed across all HER2+ patients. This association was seen for all types of ERBB2 alterations including CNV, SNV, insertion, or deletion.

Concordance between ctDNA and tumor tissue biopsy

Concordance of tumor tissue NGS and ctDNA was assessed at baseline for the most common genes at each time point (Table 2). A total of 50 patients underwent tissue NGS (29 Tempus testing, 16 Foundation One testing, 5 underwent both). Of these 50, 33 patients observed tissue sampling from a site of metastatic disease. For TP53, we observed 81.6% concordance between tissue at baseline (Cohen kappa 0.6334). In PIK3CA, we observed 81.6% (Cohen kappa 0.5890). We also observed a high level of concordance in ESR1 alterations with 81.6% (Cohen kappa 0.4159). Finally, ERBB2 was concordant in 79.6% of cases (Cohen kappa 0.3734).
Discussion

This study reports the dynamic landscape of serial ctDNA collected at times of clinical progression. We demonstrated increases to overall MAF and NOA associated with progressive disease, particularly when compared with samples from patients who did not progress. We also observed several likely resistance alterations in our cohort associated with progression, most notably TP53, PIK3CA, ESR1, AR, FGFR1, and ERBB2. Moreover, we found that MAF increased between baseline and both times of clinical progression but particularly between baseline and PN1. This is consistent with prior investigation, which showed correlation of MAF and disease burden (5, 6, 43). Between PN1 and PN2, MAF increased by a higher percentage than at PN1, however this increase was not significant. This is possibly related to the smaller sample size at PN2. When compared with patients who did not progress, patients with clinical disease progression were associated with higher MAF. At both points of clinical progression, we observed an increase in NOA that was statistically significant. When compared with patients who did not progress, patients with disease progression were associated with higher NOA. Taken together, these data point to increasing MAF and NOA as a potential marker for increasing genomic burden. This is consistent with previous investigation demonstrating an association between increased number of alterations and worse overall survival (28).

Many important prior studies have examined association between ctDNA MAF or NOA and survival. When analyzed across multiple malignancy types, increasing ctDNA has been previously
associated with disease progression, even prior to progression on imaging (23–25). Higher baseline MAF has previously been associated with worse survival (43, 44). In the BEECH trial, early suppression of ctDNA was associated with improved PFS within a validation cohort (45). Another study of ctDNA in patients with breast cancer demonstrated increasing ctDNA in patients with evidence of progressive disease (6). Our analysis of baseline MAF and NOA demonstrated an association with worse PFS in patients with MAF ≥ 3.3 and worse PFS and OS in patients with NOA ≥ 4.

Thus, our observations point to increasing MAF and NOA as possible markers of worse patient outcomes. When analyzing changes between time points, neither increasing MAF nor NOA showed any association with patient survival; however, all time points in our cohort were taken at times of progressive disease. Comparison with samples taken at times of treatment response are needed to fully characterize the association of serial changes to patient survival.

Our analysis demonstrated that across subgroups and time points, the most commonly detected alterations were TP53 and PIK3CA. This is consistent with a prior ctDNA analysis of patients with breast cancer and genomic analysis performed by The Cancer Genome Atlas Network (6, 43). TP53, in particular, showed the greatest change between time points of progression. TP53 has been previously associated with all subtypes of breast cancer, though most commonly in basal-like subtype (46). This is consistent with our cohort as 18 of the 22 patients (81.8%) with TNBC had TP53 alterations. There is evidence to suggest that TP53 may have subtype-specific prognostic implications with particularly worse prognosis in ER+ disease when compared with other subtypes with TP53 alterations (47, 48). We observed TP53 alterations at a relatively high proportion in the HR+ disease (n = 19,
Table 2. Concordance of alterations detected in blood and tissue.

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Observed</th>
<th>Kappa</th>
<th>95% CI</th>
<th>P</th>
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<tbody>
<tr>
<td>TP53</td>
<td>81.63%</td>
<td>0.6334</td>
<td>0.372</td>
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<tr>
<td>PIK3CA</td>
<td>81.63%</td>
<td>0.589</td>
<td>0.3115</td>
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<tr>
<td>ERBB2</td>
<td>79.59%</td>
<td>0.3754</td>
<td>0.0739</td>
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</tr>
<tr>
<td>EGFR</td>
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<td>0.1959</td>
<td>-0.0552</td>
<td>0.5294</td>
</tr>
<tr>
<td>ESR1</td>
<td>81.63%</td>
<td>0.4159</td>
<td>0.1063</td>
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</tr>
<tr>
<td>CCNE1</td>
<td>85.71%</td>
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<tr>
<td>MYC</td>
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<tr>
<td>NF1</td>
<td>79.59%</td>
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<td>-0.0652</td>
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</tr>
<tr>
<td>MET</td>
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<td>KIT</td>
<td>83.67%</td>
<td>-0.0829</td>
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Note: Shown are concordance of individual alterations between tissue biopsy and ctDNA at baseline.

43.2%) with increasing NOA at both time points. This suggests that TP53 may play an important role in resistance in all subtypes, but particularly in HR+ disease.

When comparing with tissue NGS, alterations detected in ctDNA at baseline showed a high degree of concordance. Of note, most of the tissue samples were taken from sites of metastatic disease suggesting that resistance alterations may arise earlier in disease progression. Another explanation is that modest to moderate increases in clonal heterogeneity may not be captured due to the presence of dominant clones found in tissue samples.

Previous studies demonstrated that PIK3CA is an important foundering mutation and driver of carcinogenesis in HR+ disease and TNBC (34, 49). Emergence of new PIK3CA driver mutations after treatment with endocrine therapy has been described previously (34). We observed an increase in the number of PIK3CA alterations between baseline and first progression, pointing to it as a possible driver of resistance as well. This increase was observed across subtypes, implying that PIK3CA may contribute to multiple mechanisms of resistance. The decreasing MAF of PIK3CA and stable NOA between PN1 and PN2 is intriguing, especially since very few patients received alpelisib (n = 3). This may suggest that while PIK3CA is an important driver of earlier progression, it may not play a critical role in later progression.

Pathogenic ESR1 mutations have been seen to arise in up to 50% of HR+ breast cancer treated with endocrine therapy (50). One prospective study showed that the use of plasma ESR1 resistance mutation to guide addition of selective estrogen receptor degraders after use of aromatase inhibitors improved progression-free survival, implicating ESR1 as a clinically useful resistance alteration (51). Our analysis confirmed the role of ESR1 as an important mechanism of resistance in the HR+ cohort. The predominance of ESR1 alterations was likely at least in part driven by the use of selective estrogen receptor degraders (fulvestrant) which have been previously associated with induction of ESR1 resistance alterations (34).

Other alterations of note in our study were FGFR1 and AR, which were both statistically associated with PN1, and ERBB2. FGFR1 increased between both time points. However, this was not statistically significant when comparing paired samples across all subtypes. FGFR1 amplifications have previously been associated with HR– disease and implicated in endocrine resistance (38, 52, 53). Our cases of FGFR1 were noted within either HR+ or HER2+ disease, which also contained cases of HR+ breast cancer. While we observed increased amplifications at progression, particularly at PN1, we also observed an increase in SNVs at both time points. These SNVs raise the possibility of other FGFR1-mediated resistance pathways, independent of previously described amplifications. AR alterations demonstrated an increase in NOA between baseline and PN1. It was predominantly associated with HR+ disease at this first time point while from PN1 to PN2, this group also included some cases of TNBC. Interestingly, AR abnormalities have previously been associated with HR+ disease and there is some prior evidence to implicate it in resistance to endocrine therapy (54–57). We also observed statistically significant change in alterations of ERBB2 (HER2) from PN1 to PN2 when analyzing paired samples by HER2+ subtype alone. However, it should be noted that we observed ERRB2 alterations across all three subtypes. ERBB2 has been known to induce chemoareistance (58–61) but has demonstrated sensitivity to anti-HER2 therapies (62).

There are several limitations of this study. First, it is a retrospective study, which is prone to known and unknown biases. This includes the pretreated nature of the cohort, making it difficult to appreciate FPS and OS, as well as varied and controlled treatments in response to ctDNA samples. Second, the sample size of patients with serial ctDNA was relatively small, particularly at PN2 (n = 27). This limits the associations between MAF/NOA and progression as well as detection of individual resistance alterations. It also limits the number of serial time points available for analysis of resistance. Third, the heterogeneity of treatments that patients received is also a limitation, as different treatments may introduce varied selective pressures to affect the types and numbers of alterations detected. Next, ctDNA alterations were not matched to whole blood cell sequencing, raising the possibility that alterations detected represent clonal hematopoiesis of indeterminate potential rather than tumor genomic evolution (63–66). Despite this limitation, bias in our findings is substantially mitigated by the high MAF of our alterations (median MAF 3%–5% depending on subgroup) as well as the high concordance of ctDNA alterations to tumor tissue NGS. In addition, many of the patients included in our study had inflammatory breast cancer (IBC). Prior studies have observed an association with IBC and an increased number of alterations compared with non-IBC (67). Several alterations were also statistically associated with IBC including TP53, ESR1, ERBB2, and FGFR3 (67, 68). Thus, the high proportion of IBC within the cohort may affect the generalizability of our findings. Finally, the temporal relationship between time point of progression and ctDNA collection also had significant variation. For example, some ctDNA collections were done on the same day as imaging and clinical assessment while other collections were done in the month preceding or following the date of clinically detected progression. This again represents the limitations of a retrospective study which is prone to variation based on individual provider practice patterns. Future studies with patients undergoing standardized treatment and ctDNA collection are needed to fully characterize resistance alterations in MBC.

In summary, we report on a large cohort of patients with serial ctDNA and clinical resistance allowing the description of molecular resistance landscape of progressive MBC. We observed an association between increasing MAF and NOA and disease progression. We noted several potentially key resistance alterations: TP53, PIK3CA, ESR1, AR, FGFR1, and ERBB2. Moreover, we detected some alterations at a higher frequency in individual subgroups, particularly ESR1 in the HR+ subgroup. Taken together, we believe these results provide a framework for the clinical utility of serial ctDNA as a tool for monitoring resistance alterations in MBC. Further prospective studies are needed to further validate and explore resistance alterations and associated response to treatment. Finally, an understanding of the biologic basis of detected resistance alterations may serve to identify novel therapeutic strategies in progressive MBC.
Serial ctDNA to Monitor Resistance in Breast Cancer

Authors’ Disclosures

A.A. Davis reports other from Merck and Lilly outside the submitted work. A.N. Shah reports personal fees from Abbvie, Daiichi Sankyo, and Taiho, as well as nonfinancial support from Pfizer outside the submitted work. K.P. Sziroskou reports other from Merck and Lilly outside the submitted work. A. Behdad reports honorarium from Foundation Medicine in China and Bayer that are not relevant to this work. A. Bardia reports grants and personal fees from Novartis, Pfizer, Genentech, Merck, Radius Health, Immunomedics (Gilead), Taiho, Sanofi, and Daiichi Pharma/AstraZeneca, as well as research funding and personal fees from Puma, Biothernostics, Phillips, Eli Lilly, and Foundation Medicine during the conduct of the study. M. Cristofanilli reports personal fees from Lilly, Pfizer, Foundation Medicine, Sermonix, G1 Therapeutics, Novartis, Menarini, and Amarex, as well as grants and personal fees from CytoDyn outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

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L. Gerratana: Conceptualization, data curation, software, formal analysis, writing—review and editing.
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The Use of Serial Circulating Tumor DNA to Detect Resistance Alterations in Progressive Metastatic Breast Cancer

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