Molecular Cancer Therapeutics

Next-Generation Sequencing of Tissue and Circulating Tumor DNA: The UC San Diego Moores Center for Personalized Cancer Therapy Experience with Breast Malignancies

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Abstract

Clinical-grade next-generation sequencing (NGS) of tissue- and blood-derived circulating tumor DNA (ctDNA) allows assessment of multiple genomic alterations in patients with cancer. We analyzed ctDNA (54–70 genes) in 62 patients with advanced breast cancer (median = five prior therapies); 38 also had tissue NGS (236–315 genes). Overall, 42 of 62 patients (68%) had detectable (characterized) ctDNA alterations (variants of unknown significance excluded), and 37 of 38 (97%) had tissue alterations. The median (range) number of characterized alterations in ctDNA was 1 (0–7), and in tissue, 4 (0–17). The most common alterations in ctDNA were in *TP53* (37% of patients) and *PIK3CA* (23%), and for tissue, *TP53* (37%) and *PIK3CA* (24%); *EGFR* amplification was seen in ctDNA (11%), but not in tissue. Concordance between ctDNA and tissue appeared higher if <6 months separated the sample acquisition, although small sample size precluded statistical validation. Overall, 32 of 67 tissue alterations (48%) were also detected in ctDNA; 35 of 72 ctDNA alterations (48%) were also in tissue. Excluding estrogen receptor and *ERBB2*, 41 of 62 patients (66%) had potentially actionable alterations in ctDNA, and 36 of 38 (95%), in tissue (with potential actionability based on either preclinical or clinical evidence). If ≥1 genomic alteration had ctDNA ≥5%, survival was shorter than if ctDNA was <5% (median, 6.7 vs. 17.9 months; $P = 0.01$). In conclusion, tissue and ctDNA NGS reveal potentially actionable alterations in most patients. The genomic results of ctDNA and tissue NGS overlap, but there are differences, perhaps reflecting temporal spacing and tumor heterogeneity. ctDNA quantification also provides prognostic information.

Introduction

The field of breast oncology was one of the first to exploit "targeted" therapy using characteristics from a patient's own tumor to direct patient care. This practice began with the use of anti-estrogen approaches with oophorectomy in the 1950's and 60's (1) and expanded rapidly with the use of agents to target hormone receptor–positive tumors in the 1980's (2). In the 1990's, targeted therapy in breast cancer changed dramatically with the discovery of *HER2* amplification (3) and the introduction of trastuzumab in 2001 (4). Targeted therapy in breast cancer has continued to grow as new therapeutic agents have been identified targeting tumors expressing hormone receptors (progestational agents, selective estrogen receptor (ER) modulators, aromatase inhibitors, selective ER degraders, and *CDK4/6* inhibitors) and tumors with *HER2* overexpression (lapatinib, pertuzumab, ado-trastuzumab-emtansine, and most recently neratinib).

Recently, in breast cancer and other solid tumors, the identification of molecular alterations in cancer-related genes that drive tumor growth, has revolutionized the field of oncology and led to a new era of precision therapy. Precision therapy requires identification of specific oncologic driver alterations, unique to an individual tumor that, when targeted, result in tumor shrinkage (5). These targeted therapies may be repurposed from FDA approvals in other malignancies or developed experimentally and tested in human clinical trials. This practice in oncology has also been designated as "personalized medicine" or "precision medicine." The benefit of this approach to cancer treatment still remains a matter of debate, although several large meta-analyses totaling about 85,000 patients indicate that a biomarker-based approach is associated with improvement in all outcome parameters (6–8), and some, but not all, prospective trials are supportive as well.

We have explored the use of two different types of next-generation sequencing (NGS) to profile patient's tumors. Initially, we began by interrogating tissue with NGS. More recently, we have also evaluated the use of "liquid" (blood) biopsies, and the assessment of NGS in circulating tumor DNA (ctDNA) in plasma. The results from both types of NGS are discussed at our weekly molecular tumor board meetings (9, 10). Herein we provide an overview of our experience with ctDNA NGS in a cohort of 62 patients with breast cancer, 38 of whom also had tissue NGS performed.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Table 1. Characteristics of patient population and characterized genomic alterations (variants of unknown significance excluded)

Total number of patients:	***N* = 62**
Gender	
Women	61
Men	1
Breast cancer receptor status	
ER+/Her2(−)	44 (71%)
ER+/Her2(+)	6 (10%)
ER(−) Her2(+)	2 (3%)
TNBC	10 (16%)
Age when tumor sent for NGS (median, range)	52 years (39–82)
Age when ctDNA sent (median, range)	55 years (40–84)
Sites of biopsies for tissue NGS (*N* = 38 patients), *N* (%)	
Liver	11 (29%)
Breast	10 (26%)
Bone	3 (8%)
Pleural fluid	3 (8%)
Brain	2 (5%)
Lung	2 (5%)
Lymph node	2 (5%)
Skin	2 (5%)
Peritoneal fluid	1 (3%)
Ovary	1 (3%)
Abdominal wall	1 (3%)
Median number of prior lines of therapy in the metastatic setting (range)	5 (0–14)
Number of patients with ≥1 characterized alteration (%)	43 (69%)
Median number of alterations/patient from tissue NGS (range)	4 (0–17)
Median number of alterations/patient from ctDNA NGS	1 (0–7)
Median number of potentially actionable alterations/patient (range)	4 (0–14)
Time between tumor tissue collection and blood collection for ctDNA (median, range)	232.5 days (3–2,371 days)

Abbreviation: TNBC, triple-negative breast cancer.

Materials and Methods

Patients

We analyzed patients seen at the University of California, San Diego Moores Cancer Center (La Jolla, CA), with advanced breast cancer who had undergone NGS of ctDNA with or without tissue NGS. This analysis and consenting procedures were in accordance with University of California San Diego Institutional Review Board (UCSD IRB, San Diego, CA) guidelines (NCT02478931). Written informed consent was obtained from all patients and all study procedures were conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. This study was approved by the UCSD IRB.

NGS

Blood-derived ctDNA. Liquid biopsy specimens were collected via standard venipuncture techniques into two tubes and sent immediately to Guardant Health (www.guardanthealth.com/guardant360/). The Guardant360 assay is an NGS assay that uses ctDNA to identify genomic alterations among 54–70 cancer-related genes (Supplementary Tables S1–S3), including 18 copy number variants (CNV), and six gene fusions. Sequencing using this method is highly sensitive and specific. The assay is able to detect >85% of single nucleotide variants (SNV) found in patients with advanced cancer with specificity of >99.9999% and detects both somatic ctDNA alterations, as well as germline alterations found in the blood stream as a result of immune cell destruction. The reported limit of detection of the Guardant360 assay is dependent on the patient's ctDNA concentration, which can range from 10 to 1000 genomic equivalents per mL of peripheral blood. The percent ctDNA was calculated by dividing the number of ctDNA SNVs by the number of wild type DNA fragments at the respective nucleotide. For CNVs detection, 1+ indicated >2.1 but ≤2.4 copy number; 2+ indicated >2.4 but ≤ 4.0; and 3+ indicated >4.0 (11, 12).

Tissue. Tumor specimens collected for tissue NGS analysis consisted of tissue either from fresh biopsies, or frozen archived tumor samples, which were sent directly to Foundation Medicine. The FoundationOne genomic assay (http://www.foundationone.com/) uses solid tumor samples from formalin-fixed, paraffin-embedded solid tumors and effusion cytology samples, to assess genetic alterations from the entire coding sequence of 236–315 cancer-related genes, plus rearrangements found in introns of an additional 28 cancer-related genes. To be reported by the FoundationOne assay, specific alterations must be identifiable in at least 10% of tumor DNA and sequencing coverage of 500× depth (13). Both companies provide clinical-grade NGS performed under Clinical Laboratory Improvement Assessment–approved standards.

Definitions

Alterations were defined as either mutation (including insertion, deletion, truncation, or rearrangement) or amplification/copy number variation. The definition of potential actionability was according to previous reports (14–16). Genes were considered theoretically actionable if the gene product was differentially expressed in tumor versus normal cells and could be targeted by a drug, or if a drug inhibited/modified the oncogenic activity of the gene product (at low IC_{50} for small-molecule inhibitors; or if the gene product was the primary target recognized by an antibody). The evidence for actionability might be based on strong clinical data or weaker preclinical data.

Table 2. Number of patients with breast cancer with potentially actionable alterations

	Tissue NGS (*N* = 38)	**ctDNA NGS (*N* = 62)**
Patients with ≥1 alteration potentially actionable by approved and/or experimental drug, *N* (%), including *ER* and *HER2*	38 (100%)	59 (95%)
Patients with ≥1 alteration potentially actionable by approved and/or experimental drug, *N* (%), excluding *ER* and *HER2*	36 (95%)	41 (66%)
Patients with ≥1 alteration potentially actionable by approved drug, (on- or off-label), *N* (%), excluding *ER* and *HER2*	34 (94%)	40 (65%)
Approved drugs(s) in another disease available, *N* (%), off-label, excluding *ER* and *HER2*	33 (87%)	38 (61%)
Experimental treatments only (clinical trials), *N* (%), (no FDA-approved treatment that impacts alteration), excluding *ER* and *HER2*	1 (3%)	2 (3%)
Patient had alteration(s) but none potentially actionable by FDA-approved drug and/or clinical trial, *n* (%), excluding *ER* and *HER2*	1 (3%)	0 (0%)
No reportable alterations, *N* (%)	1 (3%)	19 (31%)

Table 3. Potentially actionable targets and examples of their corresponding FDA-approved drugs(s)

Actionable gene	Examples of FDA-approved drug(s)	Potential actionability based on preclinical or clinical data	Comment	Reference(s)
ABL 1	Imatinib Dasatinib Bosutinib Nilotinib Ponatinib	Clinical	These drugs bind to the kinase domain of *ABL* and inhibit its activity.	30
AKT1	Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	31
AKT2	Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	31
AKT3	Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	31
APC	Sulindac	Preclinical	Sulindac is an NSAID.	32
AR	Bicalutamide Enzalutamide	Clinical	Bicalutamide and enzalutamide are androgen receptor inhibitors.	33
ATM	Olaparib	Preclinical	Olaparib is a *PARP* inhibitor.	34, 35
AXL	Cabozantinib	Preclinical	Cabozantinib is a multi-targeted kinase inhibitor.	36
BCL2	Venetoclax	Preclinical	Venetoclax is approved for the treatment of relapsed CLL.	37, 38
BRAF	Dabrafenib Vemurafenib Trametinib Cobimetinib	Clinical	Vemurafenib and dabrafenib are *BRAF* inhibitors. Trametinib and cobimetinib are *MEK* inhibitors.	39, 40
BRCA2	Olaparib	Clinical	Olaparib is a *PARP* inhibitor.	35, 41
CCND1	Palbociclib	Preclinical	Palbociclib is a *CDK4* and *CDK6* inhibitor.	42
CCND2	Palbociclib	Preclinical	Palbociclib is a *CDK4* and *CDK6* inhibitor	42
CDK4	Palbociclib	Preclinical	Palbociclib is a *CDK4* and *CDK6* inhibitor.	42
CCND3	Palbociclib	Preclinical	Palbociclib is a *CDK4* and *CDK6* inhibitor.	42
CCKN2A/B	Palbociclib	Preclinical	Palbociclib is a *CDK4* and *CDK6* inhibitor.	42
CRKL	Dasatinib	Preclinical	Dasatinib inhibits *CRKL* tyrosine phosphorylation.	43
DMNT3A	Azacitadine Decitabine	Preclinical	Azacitadine and decitabine are hypomethylating agents.	44
EGFR	Erlotinib Gefitinib Afatinib Cetuximab	Clinical	Erlotinib, afatinib, and gefitinib are inhibitors of *EGFR*. Cetuximab is a mAb that targets *EGFR*.	45, 46
ERRB2	Ado-trastuzumab emtansine Trastuzumab Pertuzumab Lapatinib Neratinib	Clinical	Trastuzumab and pertuzumab are mAbs targeting *ERRB2*. Lapatinib and neratinib are multi-kinase inhibitors that inhibit both *EGFR* and *ERRB2*.	4, 47–49
ESR1	Fulvestrant	Preclinical	Fulvestrant is a selective ER degrader.	50
FANCA	Olaparib	Preclinical	Olaparib is a *PARP* inhibitor.	35, 41, 51
FGF19	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGF19* acts via *FGFR1, FGFR2, FGFR3*, and *FGFR4.*	52
FGF3	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGF3* acts as a ligand for *FGFR1, FGFR2*, and possibly *FGFR3.*	52
FGF4	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGF4* acts as a ligand for *FGFR1, FGFR2*, and possibly *FGFR3.*	52
FGFR1	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGFR1* is a tyrosine kinase receptor.	52
FGFR2	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGFR2* is a tyrosine kinase receptor.	52
FGFR4	Lenvatinib Pazopanib Ponatinib Regorafenib	Preclinical	*FGFR4* is a tyrosine kinase receptor.	52

(Continued on the following page)

Table 3. Potentially actionable targets and examples of their corresponding FDA-approved drugs(s) (Cont'd)

Actionable gene	Examples of FDA-approved drug(s)	Potential actionability based on preclinical or clinical data	Comment	Reference(s)
FLT3	Sorafenib Sunitinib Midostaurin	Clinical for midostaurin and preclinical for other drugs	Both sorafenib and sunitinib are multi-targeted kinase inhibitors. Midostaurin is a multi-targeted kinase inhibitor that is approved for use in *FLT3*-mutated AML.	53, 54
FLT4	Sorafenib Pazopanib Sunitinib	Preclinical	*FLT-4*, also known as *VEGFR3*, is inhibited by the multi-targeted kinases listed.	55
GNAS	Trametinib	Preclinical	Trametinib is a *MEK* inhibitor.	56
IDH1	Azacitidine Decitabine	Preclinical	Azacitidine and decitabine are hypomethylating agents.	57
IDH2	Azacitidine Decitabine Enasidenib	Clinical for enasidenib and preclinical for other drugs	Azacitidine and decitabine are hypomethylating agents.	57
JAK2	Ruxolitinib Tofacitinib	Clinical	Ruxolitinib is approved for the use in polycythemia vera and primary myelofibrosis. Tofacitinib is approved for rheumatoid arthritis.	58, 59
KRAS	Trametinib	Preclinical	Trametinib is FDA approved for the treatment of melanoma and is a *MEK* inhibitor.	56
MAP2K2	Trametinib	Preclinical	Trametinib is a *MEK* inhibitor.	56
MCL1	Sorafenib	Preclinical	Sorafenib can inhibit *MCL1.*	60
MET	Crizotinib Cabozantinib	Preclinical	Crizotinib and cabozantinib are MET inhibitors.	61, 62
MSH2	Pembrolizumab Nivolumab Atezolizumab	Clinical	These drugs target PD-1 or PD-L1 and are often effective in patients with high tumor mutational burden due to mismatch repair gene defects.	63
NF1	Trametinib Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	64
NRAS	Trametinib	Preclinical	Trametinib is a *MEK* inhibitor.	56
PALB2	Olaparib	Preclinical	Olaparib is a *PARP* inhibitor.	35, 41, 51
PIK3C2B	Temsirolimus Everolimus Copanlisib	Clinical for copanlisib and preclinical for other drugs	Temsirolimus and everolimus are *mTOR* inhibitors.	31, 65
PIK3CA	Temsirolimus Everolimus	Clinical for copanlisib and preclinical for everolimus	Temsirolimus and everolimus are *mTOR* inhibitors.	31, 65
PTCH1	Vismodegib Sonidegib	Clinical	Both vismodegib and sonidegib are approved for the treatment of metastatic basal cell carcinoma.	66, 67
PTEN	Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	31, 68
RAF1	Regorafenib	Preclinical	Regorafenib is a multi-kinase inhibitor.	69
ROS1	Ceritinib Crizotinib	Clinical	Ceritinib and crizotinib are inhibitors of *ROS1.*	46, 70
RPTOR	Temsirolimus Everolimus	Preclinical	Temsirolimus and everolimus are *mTOR* inhibitors.	31
SRC	Dasatinib	Preclinical	Dasatinib inhibits *SRC*.	71
STK11	Dasatinib Everolimus Temsirolimus Bosutinib	Preclinical	Dasatinib is a multi-kinase inhibitor.	72, 73
TOP1	Irinotecan Topotecan	Preclinical	Irinotecan and topotecan are topoisomerase I inhibitors.	74, 75
TOP2	Doxorubicin Epirubicin	Preclinical	Doxorubicin and epirubicin inhibit topoisomerase II.	76
TP53	Bevacizumab Pazopanib	Preclinical	Bevacizumab (anti-*VEGF-A* antibody) has been associated with longer median PFS in *TP53* mutant than in *TP53* wild-type patients (11 vs. 5 months; retrospective study) and *TP53* mutation is associated with increased *VEGF-A*. In patients with sarcoma, pazopanib (a *VEGFR* inhibitor) response is associated with the presence of *TP53* mutations.	77–80

Abbreviation: PFS, progression-free survival.

All 54–70 genes tested in the Guardant360 assay were also tested by the FoundationOne assay except for three genes: *RHEB*, *RHOA*, and *RIT1*. All 18 genes screened for copy number variation in the Guardant360 assay were screened for copy number variation by the FoundationOne assay and all 6 gene fusions and 3 gene deletions identified by the Guardant360 assay were all present in the FoundationOne assay (11, 17).

Statistical analysis

Patient characteristics were obtained via review of the electronic medical record. Descriptive statistics were used, including medians, ranges, and frequencies, when appropriate. Overall survival (OS) was defined as the time from the ctDNA plasma collected to the date to death or last follow up date for patients still alive. Patients who were still alive were censored on their date of last follow up per chart review. The cutoff date for analysis was June, 27, 2017. OS was analyzed via Kaplan–Meier method. Statistical analysis was performed using GraphPad Prism 7.0c.

Alteration quantification and description

Standard IHC and FISH analysis of each patient's available tumor tissue pathology was used, in addition to the NGS and ctDNA results, to quantify actionable alterations in ER and HER2. For HER2/neu IHC-stained slides, positive cases are those with strong and complete membrane staining in greater than 10% of invasive cancer cells (score 3+). Negative cases are defined as those with no staining (score 0) or either weak, incomplete membrane staining in any proportion of cells or weak, complete membrane staining in less than 10% of cells (score 1+). Equivocal cases are those with weak/moderate staining intensity in greater than 10% of cells or intense, complete and circumferential staining in 10% or less of cells. HER2 testing is considered positive if there is either IHC (3+) or HER2 testing by FISH is considered amplified. FISH is considered HER2 amplified if the ratio of HER2 to CEP17 is greater than 2.2 or the average HER2 gene copy number is greater than six signals per nucleus (18, 19).

Patients that harbored multiple mutations in the same gene were not counted in our data analysis as having separate alterations, unless there was both a mutation and a copy number variation present. In the FoundationOne assay, if a genetic alteration is detected in tissue, but is not present in at least 10% of the tumor DNA, it is reported as a subclonal population. No subclonal populations were identified within our data set. If a copy number variation is found to be present in tissue, and there are six to seven alleles, it is reported as equivocal for amplification. Equivocal amplifications were included in our dataset. Eight alleles or greater are reported as amplifications. Variants of unknown significance and synonymous mutations were excluded from data analysis of both tissue and ctDNA assays.

It should be noted that when this investigation began in 2013, both the Guardant360 ctDNA and FoundationOne tissue assays evaluated slightly smaller panels of genes than when the investigation was completed in 2016. These changes in the gene panels over time were accounted for in all comparisons made between the two assays and we only performed concordance analysis of genes included in both panels.

Results

Patient characteristics

From November 2013 to March 2016, 62 patients with metastatic or locally advanced/unresectable breast cancer had ctDNA NGS performed and available for review; 38 of these patients (61%) also had tumor biopsies from either primary breast tumors or various metastatic sites sent for tissue NGS. As depicted in Table 1, all patients were women except one. The majority (71%) had hormone receptor–positive, Her2-negative breast cancer. The median patient age when tumors were sent for either tissue or ctDNA NGS analysis was 55 years. The most common site of biopsy was liver (29%) followed by breast (26%), bone (8%), and pleural fluid (8%). Patients had a median of five prior lines of therapy in the metastatic setting. Patients had a median of one characterized alteration detected by ctDNA NGS and a median of four detected by tissue NGS. A total of 69% of patients (42/62) had $\geq$1 characterized alteration detected by either ctDNA or tissue NGS. A total of 37 of 38 patients had characterized tissue alterations. The median time between collection of tumor sent for tissue NGS and collection of plasma sent for ctDNA NGS was 232.5 days (7.7 months).

Actionable mutations

As depicted in Table 2, all patients with tissue NGS (38/38, 100%) and the majority of patients with ctDNA NGS (59/62, 95%) had alterations that were theoretically actionable by either an approved or experimental drug when ER and HER2 by IHC or FISH were included as potential drug targets. When ER and HER2 were excluded from analysis, 36 of 38 (95%) of patients with tissue NGS and 41 out of 62 (66%) with ctDNA NGS had alterations that were potentially actionable by either an approved or experimental drug. However, many of the alterations were theoretically actionable based only on preclinical data (Table 3). Theoretically actionable alterations based solely on clinical data were seen in only 28 of 62 patients (45%). A total of 20 of 62 patients with ctDNA NGS results had no (characterized) alterations detected, and only 1 of 38 patients with tissue NGS had no alterations detected. A total of 54 unique genes were detected that could be considered potentially targetable by FDA-approved drugs. The specific drugs that can be used to target these genes and their mechanism of action are outlined in Table 3.

Alterations frequency

In Fig. 1A, the most frequent alterations detected by ctDNA NGS are displayed. The three most frequent ctDNA alterations involved the following genes; *TP53* (37% of patients), followed by *PIK3CA* (21%), and amplification of *EGFR* (11%). The most frequent alterations detected by tissue NGS involved the following genes: *TP53* (37%), *PIK3CA* (24%), and *GATA3* (24%) Fig. 1B. In total, alterations were detected in 29 unique genes using ctDNA NGS and in 77 unique genes using tissue NGS. The oncoprints in Fig. 1C and D and Supplementary Table S4 reveal the specific type of alterations most commonly encountered in each gene identified.

Concordance between ctDNA and tissue NGS

Of the 62 patients who had plasma sent for ctDNA NGS, 38 (61%) also had tissue sent for solid tumor NGS. Of the 38

patients with samples sent for both ctDNA and tissue NGS, 29 had at least one alteration identified by tissue NGS that was included in the ctDNA panel used at that time and could have been detected by both tissue NGS and ctDNA analysis at the time plasma was sent (only genes included in both ctDNA and tissue panels were used for concordance analysis). Of those 29 patients, 19 (66%) had ≥1 alteration identified by both ctDNA and tissue NGS assays. In Fig. 2, the concordance between ctDNA and tissue NGS detected of *TP53*, *PIK3CA*, and *ESR1* is depicted. Alterations in these 3 genes were detectable by both assays and as illustrated by the Venn diagrams. The concordance between the two assays appeared to decrease when greater than 6 months had elapsed between collection of tissue and plasma samples (although the small number of patients

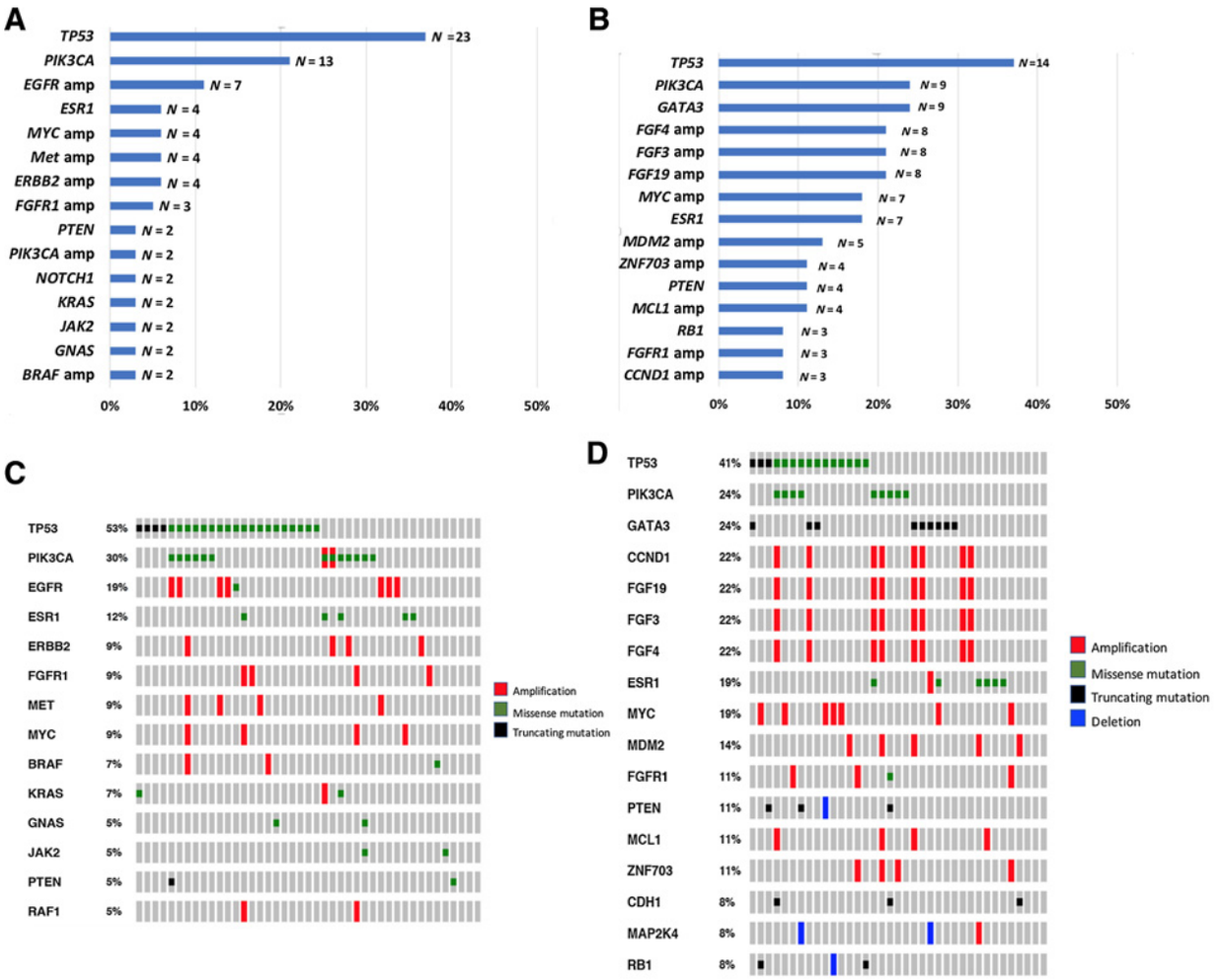

Figure 1.

A, Most frequent alterations identified by plasma-derived ctDNA NGS ($N = 62$ patients). Percent positive of patients tested is depicted. Only characterized alterations were included (synonymous alterations and variants of unknown significance were excluded.) Several patients had multiple mutations detected in the same gene; these were counted only once for each patient. On the other hand, if patients had different types of alterations in the same gene, for example, amplification in addition to mutation, these were counted as separate alterations. Genes with only one alteration noted in only 1 patient were not included in this graph; those genes were as follows: *AKT1, APC, ARID1A, ATM, CDK4* amplification (amp), *EGFR, FGFR2* amp, *FGFR3, IDH1, IDH2, KRAS, KRAS* amp, *NFE2L2, NOTCH1, RAF1* amp, *RB1,* and *STK11*. **B,** Most frequent alterations identified by tissue NGS ($N = 8$ patients). Percent of patients tested that are positive for the alteration(s) is depicted. Only characterized alterations were included (Foundation Medicine; variants of unknown significance were excluded). Several patients had multiple mutations detected in the same gene; however, multiple mutations in the same gene were only counted once per patient. On the other hand, if patients had different types of alterations in the same gene, for example, amplification in addition to mutation, these were counted as separate alterations. Equivocal amplifications (generally six or seven copies) were included. Genes with only one alteration noted in only 1 patient were not included in this graph; those genes were as follows: *AKT1, AKT2, AKT3, APC, AXIN1, BCL2L1, BRAF, C17orf39, CCND3, CDK12, CDK4, CKD8,CDKN2A/B, CEBPA, CREBBP, CRKL, CYLD, DMNT3A, ERBB2, FANCA, FGFR1, FGFR2, FGFR4, FLT3, FLT4, FRS2, GATA1, IGFR1,IKBKE. KDM5A, KDM5C, KEL, MAP2K, MDM4, MSH2, MUTYH, NFKB1A, NKX2–1, NRAS, PIK3C2B, PTCH1, RAF1, ROS1, RPTOR, RUNX1, SPTA, SRC, STAT4, STK11, TNFAIP3, TOP1,* and *TOP2*. **C,** Oncoprint of ctDNA NGS alterations ($N = 62$). Synonymous alterations and variants of unknown significance were excluded. All 62 patients were tested for ctDNA, but only 38 of them were also tested for tissue NGS (Foundation Medicine; see Materials and Methods). Each vertical bar represents a patient. **D,** Oncoprint of tissue NGS ($N = 38$). Variants of unknown significance were excluded. Each vertical bar represents a patient.

precluded statistical validation). Of the 67 alterations detected in tissue, 32 (48%) were also detected in ctDNA; of the 72 alterations detected in ctDNA, 35 (49%) were also detected in tissue (only characterized alterations analyzed in both assays are included in the calculation). Of the 11 amplifications detected in tissue, 8 (73%) were also detected in ctDNA; of the 28 amplifications detected in ctDNA, 8 (29%) were also detected in tissue (only characterized alterations analyzed in both assays are included in the calculation).

Percentage of ctDNA detected

The median percentage of ctDNA detected was 2.94% (range 0%–59.4%). We also examined the correlation between highest patient ctDNA percentage detected and OS. We observed that patients with at least one alteration with $\geq$5% ctDNA and had poorer median OS (6.7 months) versus patient with less than 5% ctDNA (17.9 months; $P = 0.01$; Fig. 3).

Discussion

This study illustrates our institution's experience with NGS of patients with breast cancer using both blood-derived ctDNA and tissue NGS to detect potentially actionable alterations. Of interest, we identified a high frequency of potentially actionable alterations using both ctDNA and tissue NGS. Overall, 66% of patients with ctDNA analysis and 95% of patients with tissue NGS had $\geq$1 alteration that could be considered actionable by and FDA-approved and/or experimental drug (even excluding ER and HER2 as targets; Table 2). However, many of the alterations were theoretically actionable based only on preclinical data (Table 3). Potentially actionable alterations based on clinical data were seen in only 28 of 62 patients (45%).

There were several notable differences between specific alterations detected by the two assays. Among the population of patients who had samples sent for both ctDNA and tissue NGS, *EGFR* amplification was detected only by the ctDNA assay and not by solid tumor NGS. This discrepancy persisted even among patients who had samples sent for ctDNA analysis and solid tumor NGS at approximately the same time (i.e., within the same week). In addition, although both assays had the ability to detect alterations in *JAK2*, this specific mutation was only detected in our patients by the ctDNA assay ($N = 2$ patients), but not in any patient by tissue NGS. This finding could be an indication of activation of the *JAK2/STAT3* pathway, which has been implicated

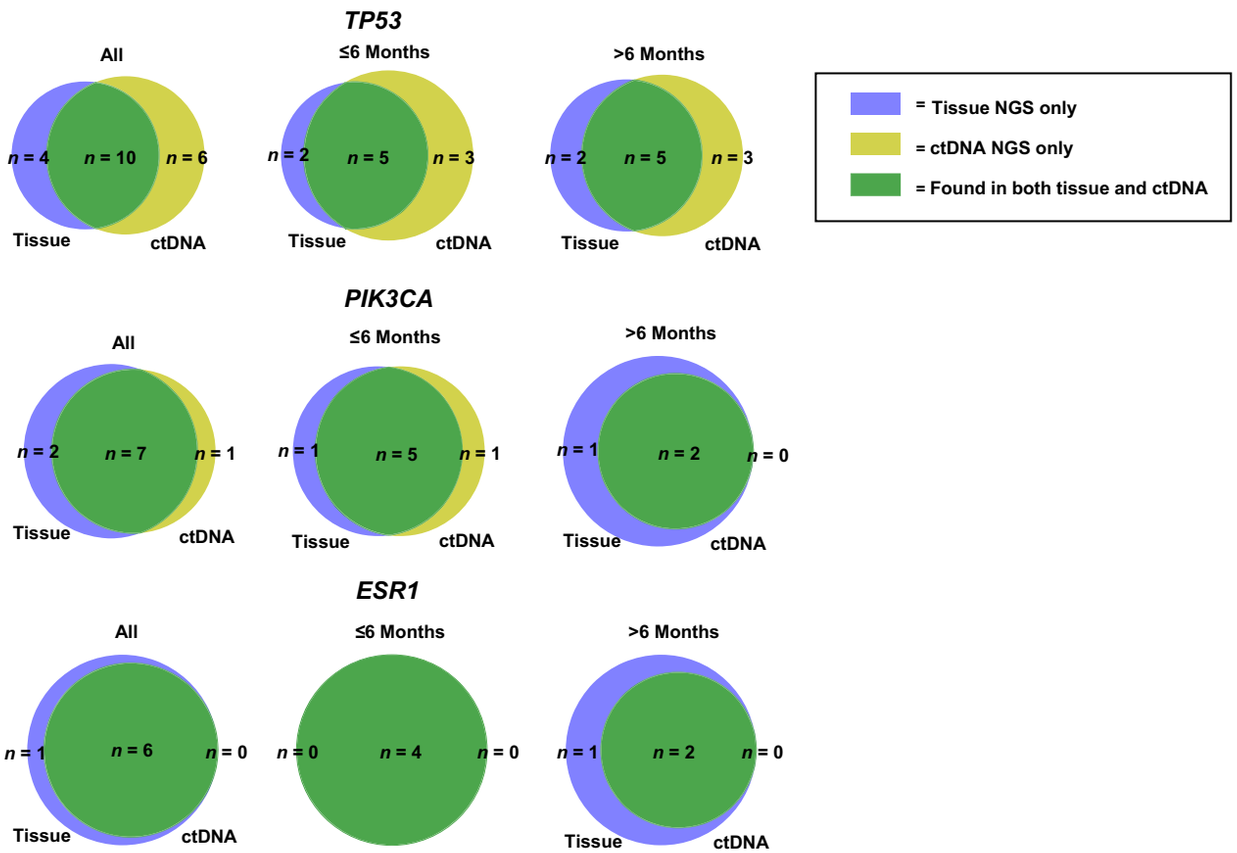

Figure 2.

Concordance between alterations identified by ctDNA and tissue NGS ($N = 38$ patients with both assays performed). Comparison depicted is the concordance in alterations detected by the two assays when tumor tissue and plasma were sent for NGS $\leq$6 months apart versus when tumor tissue and plasma were sent for NGS >6 months apart. Only alterations tested by each assay were assessed.

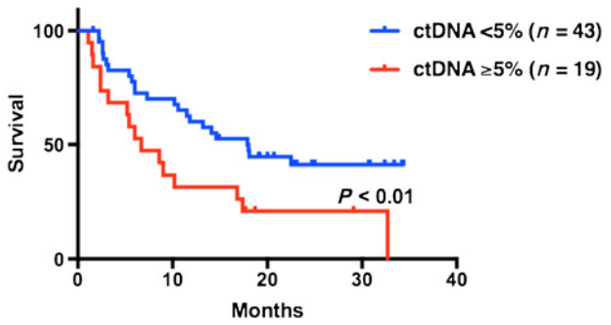

Figure 3.
OS according to the percentage of ctDNA. $N = 62$ patients available for survival analysis. Median OS for patients with ctDNA ≥5% = 6.7 months and 17.9 months for ctDNA <5% ($P = 0.01$; HR, 2.2; 95% confidence interval, 1.05–4.59; median follow-up time 11.7 months).

as a marker for breast cancer cell motility, invasion, epithelial–mesenchymal transition, and metastasis (20, 21). However, because the *JAK2* mutations were in V617F, the more likely explanation might be that this alteration reflected clonal hematopoiesis in these two elderly individuals (22). Overall, of the 67 alterations detected in tissue, 32 (48%) were also detected in ctDNA; of the 72 alterations detected in ctDNA, 35 (49%) were also detected in tissue (only characterized alterations analyzed in both assays are included in the calculation). Of the 11 amplifications detected in tissue, 8 (73%) were also detected in ctDNA; of the 28 amplifications detected in ctDNA, 8 (29%) were also detected in tissue. Our experience with the concordance between the tissue and ctDNA assays is limited by the small number of patients and the fact that different platforms were used for tissue versus ctDNA testing. However, similar concordance rates have been reported in other studies (Supplementary Table S5; refs. 23–26). As shown in Fig. 2, the concordance appears to be more robust if the sample acquisition for one assay is within 6 months of the other assay. These observations are consistent with those previously published (23, 26–29). The fact that there was less than 100% concordance observed between tissue and ctDNA NGS even if samples were taken at the same time can have several explanations: (i) suppression of ctDNA genomic alterations by specific therapies; (ii) tissue NGS reflecting the contents of the small piece of tissue tested while blood-derived ctDNA reflecting DNA shed from multiple metastatic sites; and (iii) clonal hematopoiesis that can confound ctDNA analysis. However, the largest differences between ctDNA and tissue were seen in amplifications, and technical differences or limitations in the assays could also diminish concordance.

We also performed an analysis of OS as stratified by ctDNA quantification (Fig. 3). Median survival was 6.7 months (≥5% ctDNA for at least one alteration) versus 17.9 months (each ctDNA < 5%) from the time of blood draw ($P = 0.01$). These results are consistent with those previously observed across cancer types using the same percent ctDNA cut-off value (26) and highlight the potential of ctDNA's use as a prognostic indicator.

There are several limitations to this study, the most significant of which is its retrospective nature and the small sample size. The majority of patients were hormone (estrogen)-positive and HER2-negative, and there were only small numbers of individuals in the other subtypes of breast cancer. Future studies will need to examine larger numbers of patients in each subset. Furthermore, because of the invasive nature of tissue biopsy, only 38 of 62 patients had matched tissue biopsy samples to compare with their ctDNA plasma samples, and different NGS platforms were deployed for tissue versus ctDNA interrogation. ctDNA concordance by mutant allele fraction could not be addressed because of the heterogeneity in results in this limited sample size; future studies should examine whether mutant allele fraction in tissue is predictive of percentage ctDNA for that alteration and vice versa. In addition, many patients with ER-positive, Her2-negative metastatic breast cancer have bone-only disease; bone disease is difficult to biopsy and may not be technically amenable to tissue NGS analysis. Another question of interest relates to the differences seen in metastatic versus primary tissue biopsies; unfortunately, the limited numbers of patients in this study precluded examining this question, but this warrants investigation in further studies. Finally, there have been multiple changes in the panels assayed over time, which limited our ability to do more definitive concordance analysis on all mutations detected by both assays (see Supplementary Tables S1–S3).

In conclusion, we found that the majority of patients in our population with advanced breast cancer had potentially actionable mutations identified by either ctDNA or tissue NGS, even when ER and HER2 were excluded as potential drug targets. Concordance rates between tissue and ctDNA appeared higher if the samples were obtained within 6 months of each other. However, even if samples were obtained near simultaneously, there were differences in genomic alterations detected, suggesting that tissue and ctDNA assay provide complementary results. High percentage ctDNA (≥5%) was associated with significantly poorer survival. Future prospective trials comparing and contrasting the two different technologies and evaluating how they affect patient outcomes are needed.

Disclosure of Potential Conflicts of Interest

B.A. Parker reports receiving commercial research grant from Genentech, Pfizer, Novartis, and Glaxo Smith Kline, has received speakers bureau honoraria from EMD Serono, has ownership interest (including stock, patents, etc.) from Merck, is a consultant/advisory board member for Bioatla, Inc., and has provided expert testimony for Salk Institute (patent). T. Helsten reports receiving commercial research grant from Pfizer, Synthon, Lilly, Bayer, and Novartis. R.B. Schwab has ownership interest (including stock, patents, etc.) in Samumed and has provided expert testimony for Puma Biotechnology (expert witness). R. Kurzrock is a co-founder at CureMatch, Inc., reports receiving commercial research grant from Incyte, Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, Guardant Health, Grifols, Konica Minolta, and OmniSeq, has received speakers bureau honoraria from Roche, has ownership interest (including stock, patents, etc.) in CureMatch, Inc., is a consultant/advisory board member for LOXO, X-Biotech, Actuate Therapeutics, Roche, and NeoMed, and has provided expert testimony for IDbyDNA. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Shatsky, B.A. Parker, T. Helsten, R.B. Schwab, S.G. Boles
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Shatsky, B.A. Parker, N.Q. Bui, T. Helsten, R.B. Schwab, R. Kurzrock

Writing, review, and/or revision of the manuscript: R. Shatsky, B.A. Parker, N.Q. Bui, T. Helsten, R.B. Schwab, R. Kurzrock
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Shatsky, N.Q. Bui

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