Next Generation Sequencing of Circulating Cell-Free DNA for Evaluating Mutations and Gene Amplification in Metastatic Breast Cancer

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BACKGROUND: Breast cancer tissues are heterogeneous and show diverse somatic mutations and somatic copy number alterations (CNAs). We used a novel targeted next generation sequencing (NGS) panel to examine cell-free DNA (cfDNA) to detect somatic mutations and gene amplification in women with metastatic breast cancer (MBC).

METHODS: cfDNA from pretreated patients (n = 42) and 9 healthy controls were compared with matched lymphocyte DNA by NGS, using a custom 158 amplicon panel covering hot-spot mutations and CNAs in 16 genes, with further validation of results by droplet digital PCR.

RESULTS: No mutations were identified in cfDNA of healthy controls, whereas exactly half the patients with metastatic breast cancer had at least one mutation or amplification in cfDNA (mean 2, range 1–6) across a total of 13 genes. Longitudinal follow up showed dynamic changes to mutations and gene amplification in cfDNA indicating clonal and subclonal response to treatment that was more dynamic than cancer antigen 15–3 (CA15–3). Interestingly, at the time of blood sampling disease progression was occurring in 7 patients with erb-b2 receptor tyrosine kinase 2 (ERBB2) gene amplification in their cfDNA and 3 of these patients were human epidermal growth factor receptor 2 (HER2) negative at diagnosis, suggesting clonal evolution to a more aggressive phenotype. Lasty, 6 patients harbored estrogen receptor 1 (ESR1) mutations in cfDNA, suggesting resistance to endocrine therapy. Overall 9 of 42 patients (21%) had alterations in cfDNA that could herald a change in treatment.

CONCLUSIONS: Targeted NGS of cfDNA has potential for monitoring response to targeted therapies through both mutations and gene amplification, for analysis of dynamic tumor heterogeneity and stratification to targeted therapy.

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Somatic mutation profiling of breast tumor tissues has identified a number of distinct breast cancer molecular subtypes (1–3) characterized by diverse somatic mutations, including single nucleotide variants (SNVs)4 and copy number alterations (CNAs). The 2 most frequently mutated genes are tumor protein p53 (TP53)5 and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA); however, a large number of other genes are less commonly mutated (4, 5). Genes that show amplification include erb-b2 receptor tyrosine kinase 2 (ERBB2), which can be treated with anti–human epidermal growth factor receptor 2 (HER2) agents such as trastuzumab, cyclin D1 (CCND1), fibroblast growth factor receptor 1 (FGFR1) and v-myec avian myelocytomatosis viral oncogene homolog (MYC) (6).

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Nonstandard abbreviations: SNV, single nucleotide variant; CNAs, copy number alterations; NGS, next generation sequencing; cfDNA, cell-free DNA; CA, cancer antigen; HER2, human epidermal growth factor receptor 2; SNP, single nucleotide polymorphism; ddPCR, droplet digital PCR; MBC, metastatic breast cancer; COSMIC, Catalogue of Somatic Mutations in Cancer; SIFT, scale-invariant feature transform; IGV, Integrated Genomic Viewer; VAF, variant allele frequencies; ER, estrogen receptor; PR, progestosterone receptor; RECIST, Response Evaluation Criteria in Solid Tumors; ctDNA, circulating tumor DNA; CAP, College of American Pathologists; ISO, International Organization for Standardization.

1 Human genes: TP53, tumor protein p53; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ESR1, estrogen receptor 1; ERBB2, erb-b2 receptor tyrosine kinase 2; MYC, v-myec avian myelocytomatosis viral oncogene homolog; CCND1, cyclin D1; FGFR1, fibroblast growth factor receptor 1; RPPH1, ribonuclease P RNA component H1; NOMO2, NODAL modulator; FGFR2, fibroblast growth factor receptor 2; LMTK3, lemur tyrosine kinase 3.
Targeted next generation sequencing (NGS) enables detection of low-frequency somatic mutations (i.e., SNVs detected at <5%) in heterogeneous tumor populations and in circulating cell-free DNA (cfDNA), when high coverage of >5000x (7, 8) is achieved, with the potential for guidance of treatment. However, current targeted NGS approaches that focus on key driver genes (e.g., TP53 and PIK3CA) do not fully capture genomic heterogeneity of breast cancer. Somatic CNA analysis has been carried out at the whole genome level, for example by single nucleotide polymorphism (SNP) 6.0 array (9) and low coverage sequencing (10), and ERBB2 gene amplification has been investigated by real-time quantitative PCR and droplet digital PCR (ddPCR) (11). As proof of concept, in this study we evaluated targeted NGS of cfDNA for analysis of mutations and amplification in 16 genes in 42 patients with metastatic breast cancer (MBC).

Materials and Methods

PATIENT SAMPLES, BLOOD PROCESSING, AND DNA EXTRACTION

We recruited 42 patients with radiologically-confirmed MBC (study approved by the Riverside Research Ethics Committee ref 07/Q0401/20) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue2) and 9 women attending for breast screening mammography as age-matched controls (study approved by National Research Ethics Service: 12/LO/2019). Blood sample collection was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent before participation. Twenty milliliters of venous blood was collected into EDTA-containing tubes (BD Biosciences) and 3 mL of the obtained plasma processed using the Circulating Nucleic Acids kit (Qiagen) as described previously (12). DNA was isolated from 200-μL buffy coat (for germ line DNA) and breast cancer cell lines (MDA-MB-231, MCF-7, SKBR3, and ZR-75-1) as described previously (8). Cancer antigen 15-3 (CA 15-3) results were obtained using plasma processed using the Circulating Nucleic Acids kit (Qiagen) as described previously (12). DNA was isolated from 200-μL buffy coat (for germ line DNA) and breast cancer cell lines (MDA-MB-231, MCF-7, SKBR3, and ZR-75-1) as described previously (8).

TARGETED NGS

We designed a custom 158-amplicon panel (size range 125–175 bp) across 16 genes (see online Supplemental Table 2) based on previous studies (6, 9) and publically available databases (including cBioPortal (https://www.cbioportal.org) and ArrayExpress (https://www.ebi.ac.uk/arrayexpress). A minimum of 3 ng (mean 4.8 ng) cfDNA, 5 ng lymphocyte DNA, and 1-, 5-, and 10-ng cell-line DNA was used to generate libraries. Library preparation and PGM™ sequencing (Life Technologies) were performed using the Ion AmpliSeq library prepara-

DETECTION OF SNVs AND CNAS

Sequencing data was accessed through the Torrent Suite v4.2.0, reads aligned against hg19 using Alignment v4.0-r77189, and variants called using the coverageAnalysis v4.0-r77897 and variantCaller v4.0-r76860, respectively. VariantCaller was configured to call on high stringency somatic variants with down sampling set to 2000 and the hotspot_min_allele_frequency set to 0.01 to detect very low frequency (<2%) variants. Catalogue of Somatic Mutations in Cancer (COSMIC) IDs were obtained using COSMIC v72 (13). Each cfDNA sample was compared with paired lymphocyte DNA. ANNOVAR (14) was used to annotate all variants with refGene ID, functional consequence (e.g., nonsynonymous) and functional predictions (using the scale-invariant feature transform (SIFT) (15), Polyphen-2 (16) and MutationTaster (17)). All high confidence variant calls were reviewed manually using the Integrated Genomics Viewer (IGV) package (v2.3.25) (18).

OncoCNV (19) (v6.4) was downloaded from https://oncocnv.curie.fr (accessed February 2016) and installed with Samtools (0.1.19) (20) and Bedtools (2.17.0) (21) according to the authors’ instructions. BAM files from 9 healthy controls were used to generate a cfDNA baseline control, which was compared to cfDNA BAM files from MBC with the OncoCNV default cghseg segmentation algorithm. Human genomic DNA (Roche) was used as the baseline for cell line DNA. GC content per target region was calculated using hg19 fasta (GRCh37) (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/) (accessed February 2016). A panel-wide profile plot was generated for each sample in addition to individual chromosome plots and tabular outputs. R script processSamples.R was modified to allow custom scale on the plots (available on request). A CN of ≥3 was selected as a confident threshold for gene amplification, which was above the CN ≥2.5 reported previously using ddPCR (11).

DROPLET DIGITAL PCR

Droplet digital PCR (ddPCR) was used to validate TP53, PIK3CA, and estrogen receptor 1 (ESR1) mutations as described previously (8). Primers and FAM-MGB probes were designed using Primer3 (22) for erb-b2 receptor tyrosine kinase 2 (ERBB2), v-myc avian myelocytomatisos viral oncogene homolog (MYC), NODAL modulator 2 (NOMO2), cyclin D1 (CCND1), and fibroblast growth factor receptor 1 (FGFR1) (see online Supplemental Table 3). A mean of 5 ng cfDNA (range 1.4 ng–8.5 ng) was preamplified with 12.5 μL of TaqMan preamplification master mix (Life Technologies) and 2.5
μL of a primer pool mix at 95 °C for 10 min, followed by 10 cycles of 95 °C for 15 s and 60 °C for 4 min, and a final incubation of 99 °C for 10 min. Duplex assays were run with a ribonuclease P RNA component H1 (RRPH1) (labeled with VIC-MGB) reference with 5 μL:10 dilution of premixed cfDNA, 11 μL of 2× ddPCR supermix for probes (Bio-Rad), and 1.1 μL of both target and reference assays in a final volume of 20 μL as described previously (8).

STATISTICAL ANALYSIS

Survival analysis was performed using multiple Cox-regression as described previously (23), with each biomarker as a continuous time-dependent variable. The end-of-study date was selected as December 1, 2015. Comparison of total cfDNA concentrations (copies/mL) in patients with mutation or amplification compared to those without was by t-test.

Results

We detected the expected COSMIC mutations [phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA p.E545K) in MCF-7 and ZR75–1, and TP53 p.R175H in SKBR3] and gene amplifications (MYC in MCF-7, ZR-75–1, and SKBR3 and ERBB2 in SKBR3) in cell line DNA with a mean read depth of >2000× per sample (range 2177× to 2659×) (see online Supplemental Tables 4 and 5). Dilution of the SKBR3 cell line into human genomic DNA (Roche) showed a limit of detection of 1% for TP53 p.R175H mutation and 10% for MYC and ERBB2 gene amplification, respectively (see online Supplemental Table 6). There was good agreement between results by NGS by ddPCR (see online Supplemental Fig. 1).

DETECTION OF MUTATIONS AND GENE AMPLIFICATION IN cfDNA

We sequenced paired cfDNA and lymphocytes in 42 patients with MBC (40 ERα positive, 2 ERα negative, median age 55 years; range 25–85 years; see online Supplemental Table 1) and 9 healthy female controls (median age 55 years; range 25–66 years). Mean coverage was over 1400× (range 172× to 2790×), with >360000 mean mapped reads (range 48132–626148) per sample. On average, 89% of the 158 targeted regions were covered at >500×. Only 3 amplicons, which all targeted somatic mutations, had a mean coverage of <100× and were excluded from analysis. Only 2 of 69 cfDNA samples had a mean coverage below 500×, both of which had CNA detected. No mutations or CNA were detected in cfDNA of the 9 healthy controls (see online Supplemental Fig. 2). One cfDNA leukocyte pair had a low level gain in NODAL modulator 2 (NOMO2) (CN = 2.5) within an interval that can show duplication (24).

All variants reported were detected by variant caller, with one exception, a low-frequency ESRI mutation in a serial sample from patient 4. Exactly half the patients had 1 or more gene-specific mutations and amplifications detected in cfDNA (mean = 2, range 1–6, see online Supplemental Table 7, Fig. 1A). The top 3 mutated genes in cfDNA were ESRI, TP53, and PIK3CA (Fig. 1B). Nine patients had 2 or more mutations detected and 10 patients had amplification in one or more genes (see online Supplemental Table 7). As validation, we compared NGS results with a smaller amplicon panel covering hotspot mutations in PIK3CA, TP53, ESRI, FGFR1 and fibroblast growth factor receptor 2 (FGFR2) (8) in 25 of the 42 patients. The same 9 mutations were identified across 6 patient samples and the variant allele frequencies (VAFs) were highly correlated (p = 0.9715; P < 0.0001; Fig. 1C). Mutation in the ESRI gene was significantly associated with poorer overall survival (Fig. 1D; hazard ratio (HR) 25.61; 95% CI, 4.58–143.18; P < 0.0001, log-rank test), supporting a previous study (25).

Nine patients had an HER2-positive primary tumor at diagnosis. Of these, 6 were progressing on anti-HER2 agents at the time of blood sampling, and 4 had ERBB2 gene amplification in cfDNA, whereas 3 patients who were responding to their treatment had no evidence of ERBB2 gene amplification in cfDNA (Table 1). One patient had HER2-positive metastatic biopsy and was stable on paclitaxel and herceptin at the time of blood sampling and was also negative for ERBB2 gene amplification in cfDNA. Lastly, 3 patients with an HER2 negative primary tumor had acquired ERBB2 gene amplification in cfDNA and all 3 were progressing at the time of the blood sample (Table 1).

DYNAMIC CHANGES IN cfDNA ON LONGITUDINAL FOLLOW-UP

We performed serial monitoring of alterations in cfDNA in 9 patients (Table 2, Fig. 2, and Fig. 3; also see online Supplemental Fig. 3).

Patient 1 [estrogen receptor (ER) positive, progesterone receptor (PR) positive, HER2 positive] was diagnosed with breast cancer in 2007 at age 47 years and developed metastases in November 2011. She was being treated with capcitabine and lapatinib in November 2012 at the time of the first blood sample, which had a PIK3CA mutation and gene amplification of ERBB2 and NOMO2 in cfDNA. She initially responded to therapy but relapsed in April 2013 just before the second blood sample. The PIK3CA mutation and ERBB2 gene amplification persisted; however, NOMO2 amplification was lost, suggesting a clonal shift. Total cfDNA concentrations were rising, but CA15-3 concentrations were falling with progression (Fig. 2A).

Patient 2 (ER positive, PR positive, HER2 negative) was diagnosed with breast cancer in December 2006 at
age 38 years and developed metastases in April 2012. She was progressing on goserelin and capcitabine at the time of first blood sample in May 2013, which had amplified ERBB2, TP53 mutation (VAF 6.5%), and a subclonal PIK3CA mutation (VAF 2.5%) in cfDNA, suggesting resistant disease. Her disease worsened, and she was switched to paclitaxel in April 2014. At the time of second blood sample in February 2015, she was responding to paclitaxel and the mutations resolved in cfDNA; however, total cfDNA and CA15-3 concentrations were rising and did not reflect response to treatment (Fig. 2B). Her disease progressed again in November 2015.

Patient 3 (ER positive, PR positive, HER2 negative) was diagnosed at age 48 years in 1986 and developed recurrence in her regional nodes in March 2007. She was treated with anastrozole and in April 2008 was switched to monthly fulvestrant. Computed tomography (CT) scan showed partial response by Response Evaluation Criteria in Solid Tumors (RECIST) at the time of the first blood sample in September 2012. A PIK3CA mutation was detected in cfDNA (VAF 5%) but was undetected in the second sample 14 months later, suggesting a clonal response (Fig. 2C), although total cfDNA concentrations were rising. Since then her disease has remained stable. CA15-3 concentrations were normal at both time points.

Patient 4 (ER positive, PR positive, HER2 negative) was diagnosed in 2004 at age 70 years and developed metastases in December 2010. At the time of the first blood sample in November 2014, she was stable on everolimus, exemestane, goserelin, and zolendronic acid. Mutations were detected in cfDNA in PIK3CA (VAF 11.7%) and ESR1 (VAF 10.9%). The PIK3CA mutation decreased to 1% 2 months later in January 2015. The ESR1 mutation was undetected in variant caller but was found at a frequency of 0.5% when manually inspected in IGV. Moreover, this was confirmed by ddPCR (data not shown). The coreduction in these 2 mutations suggests a clonal response (Fig. 2D), although CA15-3 concentrations were high at both time points. A solitary new liver metastasis was detected 1 month later in February.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Time between diagnosis and sample, months</th>
<th>HER2 status of primary tumor</th>
<th>Gene alteration in cfDNA</th>
<th>Treatment at time of blood sample</th>
<th>Disease status at time of blood sample</th>
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<td>10</td>
<td>16</td>
<td>Positive</td>
<td>ERBB2</td>
<td>Lapatinib and capecitabine</td>
<td>Progressing</td>
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<tr>
<td>40</td>
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<td>ERBB2</td>
<td>Vinorelbine and herceptin</td>
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</tr>
<tr>
<td>13</td>
<td>17</td>
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<td>ERBB2 and NOMO2</td>
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<td>Progressing</td>
</tr>
<tr>
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<td>Progressing</td>
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<tr>
<td>20</td>
<td>49</td>
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<td>Lapatinib and capecitabine</td>
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<td>Herceptin</td>
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<td>ERBB2 and NOMO2</td>
<td>Goserelin and capecitabine</td>
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</tr>
<tr>
<td>8</td>
<td>36</td>
<td>Negative</td>
<td>ERBB2 and MYC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tamoxifen, denosumab, anastrozole</td>
<td>Progressing</td>
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<tr>
<td>15</td>
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<tr>
<td>21</td>
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<td>PIK3CA (p.H1047R)</td>
<td>Paclitaxel and herceptin</td>
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</tr>
<tr>
<td>26</td>
<td>79</td>
<td>Negative&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NOMO2 (p.R418H)</td>
<td>Paclitaxel and herceptin</td>
<td>Stable</td>
</tr>
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</table>

<sup>a</sup> HER2 negative primary tumor, positive metastatic biopsy.

<sup>b</sup> Detected in serial sample.
Table 2. SNV/CNA tracking in 9 metastatic breast cancer patients by NGS.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Study sample, months</th>
<th>Age</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>CA15-3, U/mL</th>
<th>cfDNA genomic copies/mL</th>
<th>SNV (AA mutation) and VAF</th>
<th>VAF, %</th>
<th>Coverage</th>
<th>COSMIC ID</th>
<th>Gene amplification</th>
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<td>pos</td>
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<td>2076</td>
<td>PIK3CA p.H1047R</td>
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<td>1988</td>
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<tr>
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<td>1260</td>
<td>COSM775</td>
<td>ERBB2</td>
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<td>1260</td>
<td>COSM775</td>
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</table>

* Age is the age at the time of the first blood sample.

* pos, positive; neg, negative, NA, not applicable.
2015, and she was switched to letrozole. Her disease is currently stable.

Patient 5 (ER positive, PR positive, HER2 positive) was diagnosed in April 2012 at age 46 years and developed metastases in October 2012. Blood was collected in September and December 2014, when she was on herceptin, tamoxifen, and zoledronic acid, and CT scan showed partial response by RECIST. No alterations were detected in cfDNA at either time point, and CA15-3 concentrations were normal (Fig. 2E); her disease remains stable.

Patient 6 (ER positive, PR positive, HER2 negative) was diagnosed with MBC at age 23 years in early 2009 and relapsed in 2011 on combined endocrine therapy (fulvestrant, goserelin, letrozole, and zoledronic acid). Four blood samples were collected over a 2-year period between February 2013 and March 2015. She was being treated for brain metastases at the first sample time in February 2013, but since then and up to the time of this report is stable on combined endocrine therapy. No mutations were detected in cfDNA from the first 2 blood samples, and CA15-3 concentrations were normal. A low-frequency PIK3CA mutation was detected in the third blood sample in July 2014, which increased and was accompanied by a second subclonal mutation in TP53 and rising CA15-3 in the fourth sample in March 2015 (Fig. 2F). Although clinically stable the presence of mutations, rising concentrations of circulating tumor DNA (ctDNA) and rising CA15-3 suggest emergence of endocrine-resistant disease.

Patient 7 (ER positive, HER2 negative) was diagnosed with breast cancer in 2002 at age 61 years. She developed metastases in 2009 and was treated with sequential tamoxifen, anastrozole, exemestane, and fulvestrant. CT scan showed complete response by RECIST. At the time of the first blood sample in December 2013,
her disease had progressed and was being treated with exemestane and everolimus. No alterations were detected in cfDNA, but CA15-3 was high (314 U/mL). One month later her disease was worsening, CA15-3 rising, and a single lemur tyrosine kinase 3 (LMTK3) mutation (VAF = 3.5%) was detected in cfDNA reflecting disease progression (Fig. 2G), and she died shortly after. Based on this information an inhibitor of LMTK3 could have been used had one been available.

Patient 8 (ER positive, PR positive, HER2 negative) was diagnosed in 2004 at age 42 years and developed metastases in September 2012. At the time of the first sample in March 2014, she was stable on tamoxifen, but then progressively worsened and started denosumab and then anastrozole. Despite analyzing >3500 COSMIC mutations no alterations were detected or acquired in 3 sequential cfDNA samples during 9 months monitoring on therapy, although total cfDNA concentrations and CA15-3 were both rising. Amplification of MYC and ERBB2 genes was detected in the fourth cfDNA sample at the time of pulmonary progression (Fig. 3A), accompanied by a marked rise in cfDNA concentrations and CA15-3. She had been on capcitabine for 1 month and CT scan showed partial response by RECIST at the time fifth blood sample, MYC and ERBB2 gene amplification were undetected, and CA15-3 levels were falling. As of June 2016, her disease remains stable.

Patient 9 (ER positive, PR positive, HER2 negative) was diagnosed in November 2011 at age 40 years and developed metastases soon after in January 2012. She was initially treated with tamoxifen, which was switched to zoledronic acid, letrozole, and denosumab in November

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**Fig. 3.** Monitoring changes in gene amplification in cfDNA with treatment response. Top graphs show total cfDNA concentrations (copies/mL), ctDNA (copies/mL) and CA15-3 (U/mL). Lower graphs show gene-specific copy number detected by OncoCNV. Both patients had gene-specific amplification detected in 3 sequential cfDNA samples during 9 months monitoring on therapy, although total cfDNA concentrations and CA15-3 were both rising. Amplification of MYC and ERBB2 genes was detected in the fourth cfDNA sample at the time of pulmonary progression (Fig. 3A), accompanied by a marked rise in cfDNA concentrations and CA15-3. She had been on capcitabine for 1 month and CT scan showed partial response by RECIST at the time fifth blood sample, MYC and ERBB2 gene amplification were undetected, and CA15-3 levels were falling. As of June 2016, her disease remains stable.

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2012. She responded well and was in remission at time of the first 2 samples in May and August 2014, which showed no alterations in cfDNA, but CA15-3 concentrations were rising and remained increased (Fig. 3B). Her disease worsened over the next 6 months and paclitaxel was started. Her disease was worsening and the third blood sample in December 2014 had an ESR1 (VAF 28.6%) and subclonal PIK3CA (VAF 17.8%) mutation and CCND1 amplification in cfDNA. She started epirubicin in April 2015 was responding well at the time of sample 4 and sample 5 in June 2015 when CT scan showed stable disease by RECIST and the mutations resolved in cfDNA. She subsequently developed resistant disease and died in November 2015.

Discussion

To our knowledge this is the first targeted NGS study of cfDNA to evaluate both somatic mutations and gene specific amplification in patients with MBC. Exactly half of the 42 patients with MBC had at least one mutation or amplification in cfDNA, whereas no alterations were detected in 9 healthy female controls. The VAF ranged from 1.1% to 35.1% for point mutations in cfDNA, whereas the limit of detection for CNA was 10% cell line DNA, similar to a previous low read depth whole genome sequencing study (10), whereas a recent study focusing on SNPs detected somatic CNA with mean allelic imbalances as low as 0.5% (26). With respect to the analytical sensitivity of the assay, our results support that this panel can identify somatic variants with a VAF of ≥1% for SNVs and 5% for CNA assuming sufficient cfDNA is available for analysis. A number of other methods, including CAncer Personalized Profiling by deep Sequencing (27), Safe-Seq (28) and BEAMing (29), have reported greater analytical sensitivity for mutation detection with variant allele fractions of <0.1%. While these approaches hold great promise, a major assumption is that ctDNA is either rare or absent in the healthy, cancer-free population. Demonstrating that a ctDNA marker has a diagnostic specificity close to 100% would also be important (30). For clinical translation, detection of low-frequency variants must first be validated for CLIA/College of American Pathologists/International Organization for Standardization–based precision testing. Of importance, each assay would need to be quality controlled using well-controlled reference standards (for example, Horizon Discovery Quantitative Multiplex Reference Standards) to establish lower detection limits and reproducibility to detect variants at the lower limit.

Nine of the patients studied had more than one mutation detected in cfDNA, and in 7 patients, these mutations were at different frequencies, indicating clonal differences in the origin of circulating tumor derived DNA. Importantly, 6 patients had an ESR1 mutation detected in cfDNA while on aromatase inhibitor/endocrine therapy; this information could be used clinically to herald a change to chemotherapy. Ten patients had amplification in cfDNA in one or more genes, 3 patients had amplification only and 7 patients had both amplification and mutations. These data support heterogeneity of somatic alterations in breast cancer, with some characterized by mutations some by CNA and some by both. Ten patients had either an HER2-positive primary tumor or metastatic biopsy. Of these patients, 6 individuals had progressive disease at the time of blood sampling and 4 individuals had ERBB2 gene amplification in cfDNA, whereas ERBB2 gene amplification was undetected in 4 patients who were responding to an anti-HER2 agent. Importantly, 3 patients with a HER2-negative primary tumor had acquired ERBB2 gene amplification in cfDNA suggesting clonal evolution to a more aggressive phenotype (11, 31). This information could be used clinically to indicate that a change to an anti-HER2 therapy may be appropriate.

We performed serial monitoring in 9 patients. Results demonstrate that cfDNA profiling of mutations and amplifications could provide useful data in terms of tumor heterogeneity, clonal evolution, and response to treatment. Concentrations of ctDNA generally tracked with patient disease status, whereas when CA15-3 concentrations were high they generally remained increased. This supports the previous study by Dawson et al. (23), who suggested ctDNA to be a highly diagnostically sensitive biomarker of MBC. Interestingly, in 3 patients we saw rising total cfDNA concentrations at a time when mutations in PIK3CA and/or TP53 and ESR1 either resolved or did not increase in ctDNA. A possible explanation for this is that another clone was shedding DNA into the blood that was not characterized by any of the alterations detectable by our NGS panel. One approach to interrogate this would be to perform whole exome analysis of plasma cfDNA. Although we sequenced approximately 3500 COSMIC mutations and surveyed for amplification in 16 genes, the majority of cfDNA samples had <5 alterations detected, as has been shown in other studies using NGS mutation hot spot panels (32). This is likely due to genomic heterogeneity as there are a large number of genes infrequently mutated in breast cancers (33, 34). However, the targeted NGS approach used here has potential clinical utility where, for example, emergence of ERBB2 amplification in plasma cfDNA could signal a switch to an anti-HER2 therapy, and emergence of ESR1 mutations could indicate a switch away from endocrine therapy to standard chemotherapy. Overall, 9 patients (21%) could have been offered an alternative therapy if blood based monitoring was routine in the clinic. Our data support further investigation of the NGS approach focusing on actionable mutations and
gene amplification for monitoring treatment response and clonal dynamics in MBC.

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