Identification of somatically acquired BRCA1/2 mutations by cfDNA analysis in patients with metastatic breast cance

Neelima Vidula[*,1], **Taronish Dubash**[*,2], **Michael S. Lawrence**[3], **Antoine Simoneau**[2], **Andrzej Niemierko**[1], **Erica Blouch**[1], **Becky Nagy**[4], **Whijae Roh**[3], **Brian Chirn**[2], **Brittany A. Reeves**[2], **Giuliana Malvarosa**[1], **Jochen Lennerz**[1], **Steven J. Isakoff**[1], **Dejan Juric**[1], **Douglas Micalizzi**[1], **Seth Wander**[1], **Laura Spring**[1], **Beverly Moy**[1], **Kristen Shannon**[1], **Jerry Younger**[1], **Richard Lanman**[4], **Mehmet Toner**[5], **A. John Iafrate**[1], **Gad Getz**[1], **Lee Zou**[1], **Leif W. Ellisen**[1], **Shyamala Maheswaran**[1], **Daniel A. Haber**[1,+], **Aditya Bardia**[1,+]

[1]Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA, 02114

[2]Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA, 02129

[3]Broad Institute of Harvard and MIT, Cambridge, MA, 02139

[4]Guardant Health, Inc.

[5]Center for Engineering in Medicine, Massachusetts General Hospital, Boston, MA, 02114

Abstract

PURPOSE—Plasma genotyping may identify mutations in potentially "actionable" cancer genes, such as *BRCA1/2*, but their clinical significance is not well defined. We evaluated the characteristics of somatically acquired *BRCA1/2* mutations in patients with MBC.

METHODS—Patients with MBC undergoing routine cell-free DNA (cfDNA) next generation sequencing (73 gene panel) before starting a new therapy were included. Somatic *BRCA1/2* mutations were classified as known germline-pathogenic mutations or novel variants, and linked to clinicopathological characteristics. The effect of the PARP inhibitor olaparib was assessed *in vitro,* using cultured circulating tumor cells (CTCs) from a patient with a somatically acquired *BRCA1* mutation and a second patient with an acquired *BRCA2* mutation.

Corresponding author: Neelima Vidula, Massachusetts General Hospital, 55 Fruit Street, Bartlett Hall Extension, 1-213, Boston, Massachusetts, 02114, Phone: 617-306-4736, Fax: 617-643-0589, nvidula@mgh.harvard.edu.
[*]Equal contribution
[+]Equal contribution

RESULTS—Among 215 MBC patients, 29 (13.5%) had somatic cfDNA *BRCA1/2* mutations (nine (4%) known germline-pathogenic and rest (9%) novel variants). Known germline-pathogenic *BRCA1/2* mutations were common in younger patients (p=0.008), those with triple-negative disease (p=0.022), and they were more likely to be protein-truncating alterations and be associated with *TP53* mutations. Functional analysis of a CTC culture harboring a somatic *BRCA1*-mutation demonstrated high sensitivity to PARP inhibition, while another CTC culture harboring a somatic *BRCA2* mutation showed no differential sensitivity. Across the entire cohort, APOBEC mutational signatures (COSMIC Signatures 2 and 13) and the "BRCA" mutational signature (COSMIC Signature 3) were present in *BRCA1/2* mutant and wild-type cases, demonstrating the high mutational burden associated with advanced MBC.

CONCLUSION—Somatic *BRCA1/2* mutations are readily detectable in MBC by cfDNA analysis, and may be present as both known germline-pathogenic and novel variants.

Keywords

Introduction

Tumor genotyping is the central tenet of precision oncology and is increasingly becoming part of routine clinical care in breast cancer to identify actionable mutations for potential therapeutic intervention. However, tumor tissue genotyping of the primary tumor alone does not identify clonal evolution and mutations that are acquired during the course of treatment, some of which may be therapeutically relevant. Obtaining serial tumor biopsies from metastatic sites has been applied as proof of principle to guide successive therapeutic choices, but it is limited by the risks to the patient of an invasive procedure and accessibility of the metastatic site to biopsy, as well as biased sampling of a single tumor lesion in the midst of widespread sites of disease. Consequently, cell-free DNA (cfDNA) analyses or so-called "liquid biopsies" have emerged as an important strategy to monitor acquired cancer mutations and there has been a major rise in the clinical utilization of cfDNA assays (1–6).

While cfDNA assays are widely used in the clinic, the interpretation of multiple subclonal mutations and novel variants represents a major diagnostic challenge. This is particularly important for genes that have matched therapy approved in more traditional germline or tumor genotyping contexts, such as PARP inhibitors for patients harboring germline *BRCA1/2* mutations (7, 8). Cancer predisposing heterozygous germline *BRCA1/2* mutations, leading to somatic *BRCA*-null phenotypes, have been well studied in breast cancer (7–12), but de novo somatic *BRCA1/2* mutations are thought to be rare in breast cancer. An analysis of The Cancer Genome Atlas (TCGA) noted the prevalence of somatic *BRCA1* mutations in primary breast cancer as 1.55%, and somatic *BRCA2* mutations as 1.68% (13). However, progression to metastatic breast cancer (MBC) is associated with an increased frequency of mutations, particularly in metastatic triple-negative breast cancer (TNBC), where mutations in components of the homologous recombination pathway are more common, and the percentage of somatic *BRCA1* mutations is around 6% (14). Even in such cases of definitive acquired *BRCA1/2* mutations identified by traditional tumor

genotyping, the functional implications for sensitivity to PARP inhibition are not established. Additionally, unlike germline *BRCA1/2* genotyping, where large datasets have been curated to help interpret pathogenic and silent genetic variants within various populations, there are no such guidelines for interpreting the even more diverse potential variations in *BRCA1/2* that may be acquired somatically. These challenges are further magnified in cfDNA by the variable allele fractions and subclonal tumor cell populations that they represent. Given the general availability of cfDNA genotyping, its non-invasiveness as a diagnostic tool, and the potential for identifying impactful acquired mutations, its rapidly expanding applications require careful review before they are used to trigger therapeutic interventions.

The primary objective of this study was to understand the clinical and functional characteristics of somatic *BRCA1* and *BRCA2* mutations detectable by cfDNA in patients with MBC.

Methods

Study population

Patients with MBC who underwent cfDNA analysis as part of routine clinical care at the Massachusetts General Hospital before starting a new therapy from February 2015 to July 2017 were identified. The subset of patients with *BRCA1* and/or *BRCA2* mutations detectable by cfDNA analysis (Next generation sequencing (NGS)/Guardant360®) was determined. All consecutive patients with MBC who had Guardant360® testing during the aforementioned time interval were included, and no cases were excluded. A retrospective review of medical and pathology records, based on an IRB approved institutional protocol, was conducted to identify tumor subtype, patient demographics, germline *BRCA1/2* testing results via standard commercial germline testing and subsequent review of all cfDNA results with Guardant360® to verify the somatic nature of the mutations identified, as a post-hoc analysis, tumor genotyping results (NGS, institutional platform), and treatment outcomes post-cfDNA testing. This research was conducted in accordance with recognized ethical guidelines, including the Declaration of Helsinki, and the retrospective review was conducted based on an IRB approved institutional protocol.

CfDNA analysis

CfDNA analysis was performed using Guardant360® testing, an NGS based clinical assay evaluating 73 genes. Guardant360® employs massively parallel and deep sequencing, with an analytic sensitivity of 0.1% mutant allele fraction (MAF), with quoted specificity above 99.9%, and clinical sensitivity of 85.0% (compared to 80.7% tissue sensitivity) (15). The average molecule count is about 8000 molecules, and the average single read depth is approximately 15,000 molecules. For *BRCA1* (chromosome 17q21), exons 2–23 are included, and for *BRCA2* (chromosome 13q13), exons 2–27 are included. A retrospective chart review of the Guardant360® cfDNA reports was performed to determine the presence of somatic *BRCA1* or *BRCA2* mutations, to identify coexisting cfDNA mutations, and to characterize clonality. Based on the MAF of co-existing alterations, we defined *BRCA1* or *BRCA2* mutations as clonal (MAF ratio of *BRCA1/2* mutation/gene mutation with highest

MAF 0.25) or subclonal (MAF ratio of *BRCA1/2* mutation/gene mutation with highest MAF < 0.25) (16).

Guardant360® can identify both germline and somatic *BRCA1/2* mutations in a single test. The germline versus somatic origin of a *BRCA* mutation is determined using a decision tree algorithm, which relies on annotation from external databases (ExAC, COSMIC, ClinVar, etc) and the observed MAF of the *BRCA* variant relative to other known germline variants. All identified variants are first annotated with information from external databases to identify those variants that are known germline variants. Thereafter, variants that have an insufficient annotation to determine their origin are evaluated further based on their observed MAF relative to that of nearby known germline variants, and then a beta-binomial significance test is applied, and the variant is scored as germline or somatic. Notably, somatic *BRCA1/2* mutations are usually at a variant allele fraction two orders of magnitude lower than germline *BRCA1/2* mutations (17).

While germline results were initially suppressed in Guardant360® testing reports, as a post hoc analysis we worked with the Guardant360® team to verify the somatic nature of detected mutations.

Somatic *BRCA1/2* mutations were further classified as either known germline-pathogenic variants or as novel/unclassified variants by two independent genetic counselors, who were blinded to the Guardant360® reports. The genetic counselors evaluated the specific DNA variants seen in cfDNA, which were specifically requested from Guardant360® for this analysis. This classification was based on review of the ClinVar database (18) to identify variants that had high classification confidence (3 and 4 star review status) as of October 2018. For variants that had moderate to low classification confidence (2 stars or fewer), additional criteria such as classification reports from CLIA-certified germline genetic testing laboratories or review by consortia was evaluated. Lastly, for variants not currently in ClinVar, the likelihood of a loss of function variant (such as nonsense mutations, frameshifts, mutations in the ±1 or 2 splice site locations) was assessed, as outlined in ACMG/AMP guidelines for DNA variant classification (19). All variants not categorized as known germline-pathogenic by this analysis were then categorized as novel/unclassified, including the majority of missense mutations.

Tumor genotyping analysis

A chart review of tumor genotyping results from archival tumor tissue was performed to identify coexisting tumor mutations. An institutional NGS assay evaluating 98 genes for mutations and 91 genes for copy number changes (SNaPshot) was utilized for tumor tissue genotyping (20). This anchored multiplex PCR assay detects gene rearrangements, insertions and deletions, single nucleotide variants, and copy number changes present at allelic frequencies at 5% or higher with 100% analytical sensitivity and 100% analytical specificity (20). *BRCA1* exons 2–23 and *BRCA2* exons 2–27 are included in the assay. The time interval between cfDNA collection and the tumor tissue biopsy used for tissue genotyping was determined.

Mutation signature analysis

Mutation signatures were analyzed using Non-Negative Matrix Factorization (NMF) as described previously (21–23). Mutations detected by Guardant360® in the 29 *BRCA*-mutant patients were combined into a single "virtual patient," which was then analyzed together with 785 breast cancer patients from The Cancer Genome Atlas (TCGA). These mutation calls from whole-exome sequencing (WXS) were obtained from the TCGA Unified Ensemble "MC3" Call Set (24), the public, open-access dataset of somatic mutation calls produced by the MC3 calling effort ("Multi-Center Mutation Calling in Multiple Cancers"), downloaded from the following link: http://www.synapse.org/#!Synapse:syn7214402/wiki/405297 (The results here are in whole or part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/ as outlined in the TCGA publications guidelines (http://cancergenome.nih.gov/publications/publicationguidelines)). Joint analysis of this combined TCGA+Guardant360® dataset by NMF (k=6) revealed mutation signatures corresponding to Aging (COSMIC Signature 1), APOBEC (COSMIC Signatures 2+13), the "BRCA" signature (COSMIC Signature 3), and MSI, microsatellite instability (COSMIC Signatures 6+26). The number and fraction of mutations due to each signature was then estimated and reported.

Statistical analysis

The association between cfDNA *BRCA1/2* mutation status and patient age was determined with the Wilcoxon rank sum test, and associations with cfDNA *BRCA1/2* mutation status and tumor subtypes, prior treatment, and first treatment post-cfDNA testing were performed with the Pearson's chi-squared test. The impact of cfDNA *BRCA1/2* mutation status on progression-free survival (PFS) on the first treatment post-cfDNA testing, and overall survival (OS) was determined with the log-rank test. Cox regression analysis was used to determine the hazard ratio of cfDNA *BRCA1/2* mutation status on PFS and OS. In addition, a multi-variate analysis correcting for age and number of prior therapies was performed to determine the impact of cfDNA *BRCA1/2* mutation status on PFS and OS. For all analyses, p<0.05 was considered statistically significant.

Establishing BRCA1 mutant ex vivo circulating tumor cell (CTC) culture

A cell line (Brx401) was established from CTCs enriched from a patient with a somatic *BRCA1* mutation (in this study cohort #16). This patient had a known germline-pathogenic *BRCA1* mutation (splice site SNV ENST00000357654.3:c.5075–1G>C) detectable in the cfDNA, which was acquired after treatment with chemotherapy for metastatic TNBC. The patient had no known germline *BRCA1/2* mutation. A second CTC cell line (Brx142) was established from a patient with hormone receptor positive (HR+) MBC. In this case an early CTC culture showed wild type BRCA2, but a subsequent culture, acquired after treatment with an oral selective estrogen receptor degrader, identified a BRCA2 mutation (missense mutation E3071Q). Again, the germline testing showed no *BRCA2* mutation. Additional CTC cultures were used as controls (wild type BRCA1/2) as previously described (4). For all CTC collections, written and signed informed consent was obtained as per institutional review board approved protocol. CTCs were isolated using the microfluidic CTC i-CHIP and *ex vivo* cultures were established as described previously (4). CTC cultures were

routinely checked for mycoplasma with the MycoAlert-Lonza Kit and authenticated against matched blood sample via STR profiling by Genetica DNA Laboratories (a LabCorp brand; Burlington, NC) using the commercially available PowerPlex®16HSamplification kit (Promega Corporation; mouse marker included) and GeneMapper ID v3.2.1 software (Applied Biosystems).

Whole-exome sequencing

For whole-exome sequencing (WES), the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) was used for extraction of genomic DNA. DNA was quantified in triplicate using a standardized PicoGreen® dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA) assay. The quality control identification check was performed using fingerprint genotyping of 95 common SNPs by Fluidigm Genotyping (Fluidigm, San Francisco, CA). Library construction was performed using the KAPA Library Prep kit, with palindromic forked adapters from Integrated DNA Technologies. All library construction, hybridization and capture steps were automated on the Agilent Bravo liquid handling system. Flowcells were sequenced utilizing Sequencing-by Synthesis chemistry for HiSeq 4000 flowcells. Each pool of whole exome libraries was sequenced on paired 76 cycle runs with two 8 cycle index reads across the number of lanes needed to meet coverage for all libraries in the pool (raw data available on request).

Somatic mutation calling from whole exome sequencing (WES) data

Exome sequencing data of CTC lines were used to identify somatic single nucleotide variations (sSNVs) and somatic small insertions and deletions (sINDELs). Output from Illumina software was processed by the Picard and GATK toolkits developed at the Broad Institute. The BAM files were generated by aligning with bwa version 0.5.9 to the NCBI Human Reference Genome Build hg19. Prior to variant calling, the impact of oxidative damage (oxoG) to DNA during sequencing was quantified as described previously (25). The cross-sample contamination was measured with ContEst (26) based on the allele fraction of homozygous SNPs, and this measurement was used in MuTect. From the aligned BAM files, somatic alterations were identified using a set of tools developed at the Broad Institute (www.broadinstitute.org/cancer/cga). The details of the sequencing data processing have been described previously (27, 28). Following our standard procedure, sSNVs were detected using MuTect (version 1.1.6) (28); sINDELs were detected using Strelka (version 1.0.11) (29). Then an allele fraction specific panel-of-normals (PoN) filter was applied to filter false positive germline variants and common artifacts from mutation calls, which compares the detected variants to a large panel of normal exomes or genomes and removes variants that were observed in the panel-of-normals. All somatic mutations, insertions and deletions were annotated using Oncotator (version 1.4.1) (30) sSNVs and sINDELS in only cancer genes (Cancer Gene Census) (31) were used for mutation status analysis.

Olaparib sensitivity studies

Three independent breast cancer CTC lines were tested for drug sensitivity: Brx401, harboring a somatically acquired known germline-pathogenic *BRCA1* mutation, Brx142, harboring a somatically acquired mutation in *BRCA2*, and Brx07, with wild type *BRCA1/2* alleles. CTC lines were seeded in 96 well ultra-low attachment plates (Corning) at 1000 cells

per well. Increasing concentrations of olaparib (Selleckchem S1060) ranging from 0.01μM to 50μM were added to quadruplicate wells. Cell viability was measured using CellTiter-Glo Luminescent Cell Viability Assay per the manufacturer's instructions at day 5.

Immunoblot

Cell pellets were lysed in 100mM Tris pH 6.8 1% SDS, sonicated for 10 seconds using a 4710 Series Ultrasonic Homogenizer (Cole-Parmer) and incubated for 3 min at 95°C. Protein lysates were then quantified and normalized using Pierce BCA Protein Assay Kit (23227, ThermoFisher Scientific). Lysates were then combined 1:1 with 2x sample buffer (100 mM Tris pH 6.8, 12% glycerol, 3.5% SDS, 0.2M DTT) and 20 μg of protein was loaded onto 4–12% Bolt Bis-Tris Plus gels (NW04122BOX, ThermoFisher Scientific) and transferred onto PVDF membranes by liquid transfer with CBS Scientific Electrophoretic Blotting System (EBX-700, 100V, 2 hours). Membranes were immunoblotted using *BRCA1* (1:1000, D-9, Santa Cruz), GAPDH (1:20000, AB516, Millipore) and H3 (1:40000, ab1791, Abcam) antibodies and HRP-conjugated secondary anti mouse (1:5000, 115–035-003, Jackson Immunoresearch) and anti-rabbit (1:5000, 111–035-003, Jackson Immunoresearch) antibodies. Signals were detected using the Chemidoc imaging system (Bio Rad) with Image Lab v6.0.1 software.

Results

Patient demographics

We identified 215 patients at the Massachusetts General Hospital with MBC who had undergone cfDNA analysis before the start of a new therapy (first-line or greater) from February 2015 to July 2017. Supplemental figure S1 provides a consort diagram delineating the study population. Of the total population with MBC, 29 (13.5%) had somatic *BRCA1* or *BRCA2* mutations detectable by cfDNA. In nine patients (4.2%) mutations previously described as known germline-pathogenic were detected (as described in Supplemental Table S1), while in 20 (9.3%) novel variants (not previously reported in public databases) were identified (18).

Altogether, patients with cfDNA *BRCA1* or *BRCA2* mutations had similar age, cancer subtype distribution, and number of prior lines of chemotherapy, and went on to receive similar therapies post testing as those lacking cfDNA *BRCA1/2* mutations (*BRCA* WT population) (supplemental Table S1). The majority of patients with somatic *BRCA1/2* mutations had MBC which was recurrent (97%), rather than de-novo. The characteristics of patients with either known germline-pathogenic *BRCA1/2* somatic mutations or novel variants are shown in Table 1. Interestingly, the patients with known germline-pathogenic *BRCA1/2* mutations were significantly younger (median age of 48 years vs. 55 years) (p=0.008) and more often had TNBC (44% vs. 5%) (p=0.022), compared with the novel variants which were generally seen in HR+ and in some HER2+ MBC. The analyses of somatic *BRCA1/2* status on patient outcomes is described in the supplemental section and supplemental Figure S2.

Characteristics of cfDNA BRCA1/2 mutations

There was significant heterogeneity in the type of mutation and clinico-genomic characteristics of somatic *BRCA1/2* mutations as depicted in Table 2. Four patients (13.8%) had polyclonal (2) *BRCA1/2* mutations and 3 patients (10.3%) had both *BRCA1* and *BRCA2* cfDNA mutations.

Among the various *BRCA1/2* mutations detected in the cohort, 11 (29%) were protein-truncating alterations (6 frameshift insertions/deletions, 2 splice variants, and 3 nonsense mutations), all of which were predicted to be pathogenic. In contrast, 20 (53%) were missense point mutations, the majority of which were novel variants of unknown significance.

Altogether, 45.7% of the detected mutations were clonal (i.e. MAF ratio 25%) and 54.3% were subclonal (MAF ratio <25%). In the entire *BRCA1/2* mutant population, 62% of patients with a cfDNA *BRCA1/2* mutation had received prior platinum and/or anthracycline therapy, and this treatment distribution was similar in patients with known germline-pathogenic mutations. However, fewer patients with novel variants had received prior anthracycline or platinum therapy.

Of the 29 patients with somatic *BRCA1* or *BRCA2* mutations, 21 had archival tumor available for analysis (details in supplemental section, 52.4% on metastatic lesion at MBC diagnosis, 33.3% on a metastatic lesion post MBC diagnosis, and 14.3% on primary tumor specimen). Out of 21, only 3 patients had somatic *BRCA1* mutations detectable in the archival tumor tissue, all from metastatic specimens. The *BRCA1* variants in these 3 cases were identical in the blood and metastatic tumor tissue (details in supplemental section). The detailed clinical history and timing of tissue versus blood genotyping is outlined in supplemental Table S2.

One patient (patient ID #17) had a known coexisting germline *BRCA1* mutation (c. 3875del4 mutation) as well as 3 additional somatic *BRCA1* mutations in exon 10 which appeared to be reversion mutations, restoring the open reading frame in different ways. This patient had received platinum chemotherapy, which may have triggered the development of the *BRCA1* reversion mutations that can restore BRCA1 function leading to acquired resistance to platinum and/or PARP inhibitors (32, 33).

Coexisting cfDNA mutations

As depicted in Figure 1, a wide spectrum of coexisting mutations were detected with somatic *BRCA1/2* mutations, highlighting genomic complexity and clonal heterogeneity. The most common mutations included *PIK3CA* (44.8%), *TP53* (41.4%), *NF1* (27.6%), *ERBB2* (20.7%), *MET* (17.2%), *ARID1A* (17.2%), *EGFR* (13.8%), *APC* (13.8%), *NOTCH1* (13.8%), *RHOA* (10.3%), *ESR1* (10.3%), *KIT* (10.3%), and *FGFR3* (6.9%).

TP53 mutations were more common among patients with known germline-pathogenic *BRCA1/2* mutations (77%), as compared to patients with novel *BRCA1/2* variants (30%). Supplemental Figure S3 depicts the mutation spectrum by MAF for each patient in this cohort.

In terms of mutation signatures, comprehensive analysis revealed that both the "BRCA" signature (COSMIC Signature 3, associated with homologous recombinant deficiency) as well as APOBEC mutational signatures (COSMIC Signatures 2+13) were present in the *BRCA1/2* cohort, highlighting the functional heterogeneity with somatic *BRCA* mutations (Figure 2).

BRCA protein expression and olaparib sensitivity in CTC-culture lines

Finally, to evaluate the functional significance of somatic *BRCA1* mutations, we analyzed gene expression and BRCA1 protein expression in the CTC lines Brx401 (harboring a known germline-pathogenic somatic *BRCA1* mutant derived from patient ID #16 in this cohort) and Brx07 (harboring *BRCA1* WT) using Western Blot (Figure 3A). Additional coexisting mutations in Brx401 included TSC2, TP53, and NOTCH2. No BRCA1 protein was seen in the cell line with a somatic *BRCA1* mutation (Brx401) highlighting functional loss of BRCA1 protein, but full-length BRCA1 protein was seen in the cell line harboring *BRCA1* WT (Brx07). In addition, we treated the Brx401 and Brx07 CTC cell lines with olaparib for 5 days and evaluated cell proliferation. The known germline-pathogenic somatic *BRCA1* mutant line (BRx401) demonstrated increased sensitivity to olaparib (IC50 6.48μM) compared to the *BRCA* WT line (BRx07) (IC50 63.68μM) (Figure 3B). Indeed, the patient (patient ID #16 from whom the CTC-culture line BRx401 was developed) derived therapeutic benefit with carboplatin (PFS approximately 6 months), but not eribulin (PFS 3 months), further confirming that the somatic *BRCA1* mutation was a likely driver mutation and consistent with the known platinum sensitivity of pathogenic *BRCA1/2* mutations.

Furthermore, in a third breast cancer CTC line harboring a novel somatic *BRCA2* variant (Brx142) from a patient with HR+ metastatic breast cancer, we observed no increased sensitivity to olaparib compared with cells with *BRCA* WT. Interestingly, in this line, an APOBEC mutational signature was widely evident, and it encompassed the novel *BRCA2* mutation itself. Additional coexisting mutations observed included *SMARCA4* (p.1787M and p.E1606Q), *CIC* (p.E2258Q and p.K2423N), *PI3KCA*, *BCLAF1*, *FAM135B*, *ALK*, *CSMD3*, *MYCN*, *FAT1*, *NF2*, *MUC16*, *MAFB*, *ZNF331*, *APC*, and *HIF1A*, but a *TP53* mutation was not present in this line. Thus, the somatic *BRCA2* variant is likely a passenger mutation induced by increased APOBEC activity.

Discussion

We report that a proportion of patients with MBC harbor somatically acquired *BRCA1/2* mutations in cfDNA, but that there is significant diversity in their associated clinico-genomic characteristics. While some of these mutations may be pathogenic in nature, others may not have functional significance. We were able to showcase the differences among these in selected cases for which cultured CTCs could be generated, but in general, distinguishing between cases with pathogenic *BRCA1/2* mutations likely to respond to PARP inhibition and those with passenger mutations will require careful interpretation of both mutational and clinical parameters, and ultimately confirmation in prospective clinical trials. We identified that many detected mutations are subclonal. The clinical utility of using PARP inhibition to treat subclones with acquired *BRCA1/2* mutations within a heterogeneous cancer is not

known. The advent of PARP inhibitors as an approved therapy for germline *BRCA1/2* mutant advanced breast cancer and the efficacy of DNA damaging agents in *BRCA1/2* germline mutant patients makes the identification of nonfamilial cases with tumors that have *BRCA*-like features important, since this may help extend the application of PARP inhibitors (7, 8, 34), as has been demonstrated in ovarian cancer where germline and somatic *BRCA* mutant tumors have similar responses to PARP inhibition and platinum salts (35–43).

We identified that 13.5% of patients with MBC had somatic *BRCA1* or *BRCA2* mutations detectable by cfDNA. This mutation frequency is higher than expected based on the rates of somatic *BRCA1/2* mutations in primary breast cancer (13), and possibly reflects the acquisition of mutations under therapeutic pressure (3, 4, 44). The majority of the *BRCA1/2* mutations detected by cfDNA are not presently known to be pathogenic, and rather were novel variants, some of which might be passenger mutations resulting from APOBEC activity and other mutagenic conditions (14, 23, 45). A limitation of the Guardant360® assay used in these analyses is that tumor mutation burden cannot be calculated so we could not determine whether the presence of cfDNA *BRCA1/2* mutations may be linked to an increased mutation rate, although more sophisticated cfDNA assays to evaluate this association could be considered in the future.

There are presently no guidelines to determine the pathogenicity of somatic *BRCA1/2* mutations. Based on our work, we advocate the approach summarized in Figure 4 to determine the pathogenicity of a somatic *BRCA1/2* mutation detected in cfDNA prior to consideration of PARP inhibitor therapy. We recommend initial review of detected somatic *BRCA1/2* mutations by expert genetic counselors, utilizing open databases such as ClinVar (18) to understand the functional impact of genetic variants including splice site changes, frameshifts, stop codons, and missense mutations at different locations within the *BRCA1/2* coding sequence. We recognize that this approach is limited by the uncertainty of extrapolating pathogenic mutation status from germline to somatic sequence analysis, and the fact that many mutations detected are likely to be novel variants whose functional significance is not known. The prospective development of large somatic genomic databases will be helpful in future classification. Clinical characteristics may help determine the potential for a somatic *BRCA1/2* mutation to be pathogenic, such as TNBC histology and young age at diagnosis, criteria that are also characteristic of pathogenic germline *BRCA1/2* mutations (46, 47). In contrast, most of the HR+ cases with cfDNA *BRCA1/2* mutations were novel variants of uncertain significance. The coexisting genomic environment may provide clues such as coexisting *TP53* mutations which we observed more commonly in patients with pathogenic *BRCA1/2* mutations, similar to the association between *TP53* mutations and germline *BRCA1/2* mutations (48). While these criteria may provide guidance in interpreting cfDNA *BRCA1/2* mutations, ultimately prospective clinical trials of PARP inhibitors in nonfamilial breast cancer are needed (49, 50).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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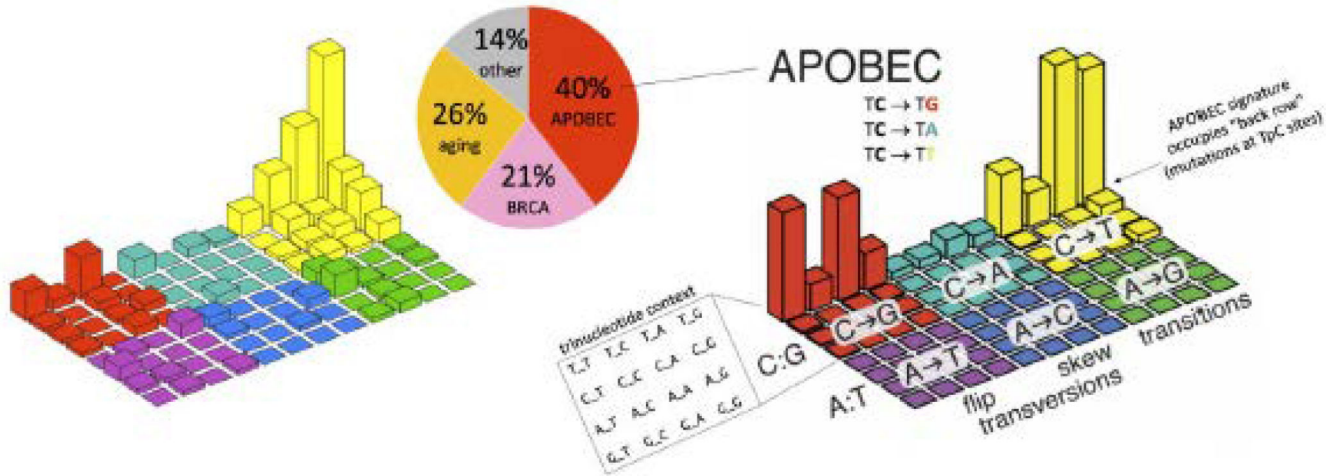
Statement of Translational Relevance

Identification of somatic mutations using plasma genotyping assays in patients with metastatic breast cancer (MBC) represents an opportunity for novel therapy selection, and a challenge in distinguishing clinically impactful genetic variants. For *BRCA1/2* mutations, pathogenic germline mutations are well-annotated, whereas the therapeutic significance of somatically acquired variants is not well-defined. We describe a cohort of patients with MBC, in whom we identified *BRCA1/2* mutations using cell-free DNA (cfDNA) genotyping, with clinical correlates, and in selected cases conducted functional assays in cultured circulating tumor cells (CTCs). As many as 13.5% of patients with MBC harbor somatic *BRCA1/2* mutations in cfDNA; 4% are known germline-pathogenic variants. In CTC-derived models, certain cell lines with somatically acquired driver variants demonstrate increased sensitivity to PARP inhibitors, while others with somatic *BRCA1/2* variants resulting from increased APOBEC-mediated mutagenesis do not, behaving as passenger mutations. Detection of *BRCA1/2* mutations using cfDNA requires caution before PARP inhibitor application.

Figure 1. Coexisting cfDNA mutations in patients with MBC with cfDNA *BRCA1/2* mutations.
Blue depicts wild-type genes, yellow denotes known pathogenic mutations, and green signifies novel variants.

Figure 2. APOBEC mutation signature in somatic *BRCA*-mutant patients.
The "Lego" plot on the left is the study data (all somatic mutations in the cohort). The "Lego" plot on the right is a "reference" for comparison: the APOBEC mutation signature. It also has the plot axes labeled. The rows are not mutational signature, but rather the whole plot is a mutation signature. The APOBEC mutation signature was clearly observed in this cohort. Approximately 40% of mutations were assignable to the APOBEC mutation signature (back row of bars in the "Lego" plot, COSMIC signatures 2+13), summing mutations across the 29 patients that were found to carry somatic *BRCA* mutations. Other contributors to the mutations in these *BRCA*-mutant patients were the "aging" signature (COSMIC Signature 1) and the "BRCA" signature (COSMIC Signature 3).
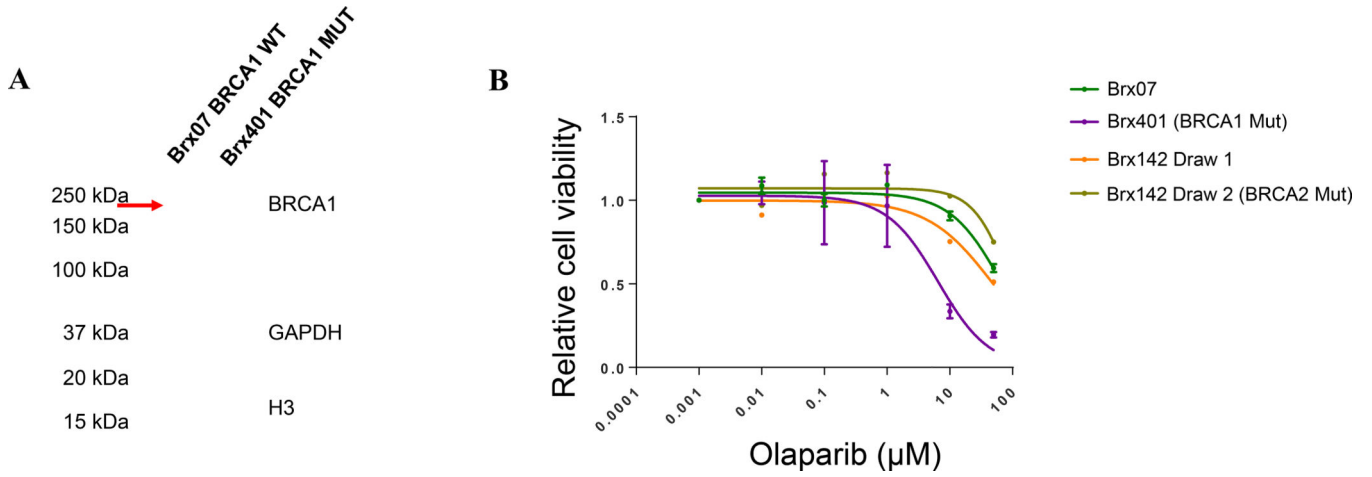
Figure 3. BRCA protein expression and olaparib sensitivity in CTC-culture lines.
(A) CTC lines Brx401 (acquired somatic *BRCA1* mutant, MUT) and Brx07 (wild-type, WT) were analyzed by Western blot for BRCA1 protein expression. No BRCA1 protein was detected in Brx401. The red arrow indicates full-length BRCA1 protein (220kDa) detected in Brx07. (B) Brx401, Brx07, and BRx142 (novel variant *BRCA2* mutant acquired (Brx142 (Draw 2) from baseline Brx142 (Draw 1) after treatment with a serum estrogen receptor degrader) CTC cell lines were treated with increasing concentrations of olaparib for 5 days and cell proliferation was evaluated. Brx401 (acquired somatic *BRCA1* mutant, IC50: 6.48μM) was more sensitive to PARP inhibition compared to BRx07 (wild-type, IC50: 63.68μM) and the Brx142 lines.

Somatic *BRCA1/2* mutation detected in cfDNA (non germline *BRCA1/2* carrier)

Step 1: Determine functional impact on BRCA1/2 protein with assistance of genetic counselors by evaluating specific DNA variants with databases such as ClinVar[18] and comparison to germline testing reports from CLIA certified laboratories.

Step 2: Evaluate clinical characteristics such as younger patient age and the presence of triple-negative breast cancer that may support the presence of a pathogenic *BRCA1/2* mutation.

Step 3: Evaluate the coexisting genomic landscape for other mutations such as *TP53* that may be more suggestive of a pathogenic *BRCA1/2* mutation.

Future goals:
1. Real time CTC culture for individualized preclinical testing of individual *BRCA1/2* variants
2. cfDNA mutation-specific information from large prospective clinical trials

Figure 4. Approach to establishing the pathogenic nature of a somatic *BRCA1/2* mutation in cfDNA.
Based on our work, we recommend determining the functional impact on the BRCA1/2 protein (step 1), using clinical characteristics and the coexisting genomic landscape (steps 2–3) to help corroborate the presence of a pathogenic mutation. Future goals to aid in this assessment include developing real time CTC culture for individualized preclinical testing of individual *BRCA1/2* variants, and obtaining data on the utility of PARP inhibition for various cfDNA *BRCA1/2* mutations from large prospective clinical trials.

Table 1.

Clinical characteristics of patients with metastatic breast cancer and known germline-pathogenic *BRCA1/2* mutations or novel *BRCA1/2* variants.

Clinical Variable	cfDNA *BRCA1/2* mutation absent (*BRCA* wild-type (WT)) (N=186)	cfDNA *BRCA1/2* known germline-pathogenic mutation present (*BRCA* known germline-pathogenic mutant)* (N=9)	cfDNA *BRCA1/2* novel variant mutation present (*BRCA* novel variant)* (N=20)	p value for difference between *BRCA* WT and *BRCA* known germline-pathogenic mutant[+]	p value for difference between *BRCA* known germline-pathogenic mutant and *BRCA* novel variant[+]
Median age at metastatic breast cancer diagnosis	57 (48–65)	48 (46–52)	55 (52–67)	0.059	0.008
Tumor Subtype				0.023	0.022
HER2+	11 (5.9%)	0 (0%)	3 (15%)		
HR+	134 (72%)	4 (44.4%)	14 (70%)		
TNBC	24 (12.9%)	4 (44.4%)	1 (5%)		
Unknown	17 (9.1%)	1 (11.1%)	2 (10%)		
Number of prior lines of chemotherapy				0.98	0.26
0–1	124 (66.7%)	6 (66.7%)	17 (85%)		
2	61 (32.8%)	3 (33.3%)	3 (15%)		
Unknown	1 (0.5%)	0 (0%)	0 (0%)		
First therapy post-cfDNA testing				0.32	0.42
Endocrine	57 (30.6%)	3 (33.3%)	7 (35%)		
HER2 therapy	13 (7.0%)	0 (0.0%)	2 (10%)		
Immunotherapy	14 (7.5%)	2 (22.2%)	2 (10%)		
Chemotherapy	49 (26.3%)	3 (33.3%)	3 (15%)		
Other	36 (19.4%)	0 (0.0%)	3 (15%)		
None	13 (7.0%)	0 (0.0%)	2 (10%)		
Unknown	4 (2.2%)	1 (11.1%)	1 (5%)		

*
Patients with both known germline-pathogenic and novel variants present in cfDNA were included in the known germline-pathogenic category for these analyses.

[+]
For the statistical analyses, the Wilcoxon rank-sum test (age variable) and Pearson chi-squared test (all categorical variables) were used.

Table 2.

Characteristics of cfDNA *BRCA1/2* mutations.

Characteristics of cfDNA *BRCA1/2* mutations (N=29, overall cohort)	
Characteristic	**Number of patients**
BRCA1 or *BRCA2*	*BRCA1*: 15 (51.7%) *BRCA2*: 11 (37.9%) Both *BRCA1* and *BRCA2*: 3 (10.3%)
Previously known germline-pathogenic vs. novel variants	Known germline-pathogenic: 9 (31%) Novel variants: 20 (69%)
Clonal vs. subclonal	Clonal: 16 (45.7%) Subclonal: 19 (54.3%)
Prior platinum or anthracycline treatment before cfDNA testing	Prior platinum: 4 (13.8%) Prior anthracycline: 16 (55.2%) None: 11 (37.9%)
Coexisting germline *BRCA1/2* mutation	Germline *BRCA1* mutation: 1 (3.4%) Germline *BRCA2* mutation: 0 (0%) No known germline *BRCA1* or *BRCA2* mutation: 28 (96.6%)
Coexisting *BRCA1/2* mutation detectable by tumor tissue genotyping	*BRCA1*: 3 (15.8%) *BRCA2*: 0 (0%) No *BRCA1* or *BRCA2* mutation detected by tumor tissue genotyping: 16 (84.2%)
Characteristics of cfDNA previously known germline-pathogenic *BRCA1/2* mutations (N=9) [*]	
BRCA1 or *BRCA2*	*BRCA1*: 5 (55.6%) *BRCA2*: 3 (33.3%) Both *BRCA1* and *BRCA2*: 1 (11.1%)
Clonal vs. subclonal	Clonal: 4 (44.4%) Subclonal: 5 (55.6%)
Prior platinum or anthracycline treatment before cfDNA testing	Prior platinum: 4 (44.4%) Prior anthracycline: 5 (55.6%) None: 2 (22.2%)
Coexisting germline *BRCA1/2* mutation	Germline *BRCA1* mutation: 1 (11.1%) Germline *BRCA2* mutation: 0 (0%) No known germline *BRCA1* or *BRCA2* mutation: 8 (88.9%)
Coexisting *BRCA1/2* mutation detectable by tumor tissue genotyping	*BRCA1*: 2 (40%) *BRCA2*: 0 (0%) No *BRCA1* or *BRCA2* mutation detected by tumor tissue genotyping: 3 (60%)
Characteristics of cfDNA novel variant *BRCA1/2* mutations (N=20) [*]	
BRCA1 or *BRCA2*	*BRCA1*: 10 (50.0%) *BRCA2*: 5 (25.0%) Both *BRCA1* and *BRCA2*: 5 (25.0%)
Clonal vs. subclonal	Clonal: 10 (50.0%) Subclonal: 10 (50.0%)
Prior platinum or anthracycline treatment before cfDNA testing	Prior platinum: 0 (0%) Prior anthracycline: 11 (55.0%) None: 9 (45.0%)
Coexisting germline *BRCA1/2* mutation	Germline *BRCA1* mutation: 0 (0%) Germline *BRCA2* mutation: 0 (0%) No known germline *BRCA1* or *BRCA2* mutation: 20 (100%)
Coexisting *BRCA1/2* mutation detectable by tumor tissue genotyping	*BRCA1*: 1 (6.7%) *BRCA2*: 0 (0%) No *BRCA1* or *BRCA2* mutation detected by tumor tissue genotyping: 14 (93.3%)

[*] For these analyses, patients with both known germline-pathogenic and novel variants in cfDNA were included in the known germline-pathogenic category.