A Targeted Next-Generation Sequencing Assay Detects a High Frequency of Therapeutically Targetable Alterations in Primary and Metastatic Breast Cancers: Implications for Clinical Practice

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Next-generation sequencing • Precision medicine • Molecularly targeted therapy • Predictive markers

ABSTRACT

Background. The aim of this study was to assess the frequency of potentially actionable genomic alterations in breast cancer that could be targeted with approved agents or investigational drugs in clinical trials using a next-generation sequencing-based genomic profiling assay performed in a Clinical Laboratory Improvement Amendments-certified and College of American Pathologists-accredited commercial laboratory.

Methods. Fifty-one breast cancers were analyzed, including primary tumor biopsies of 33 stage I–II and 18 stage IV cancers (13 soft tissue, 3 liver, and 2 bone metastases). We assessed 3,230 exons in 182 cancer-related genes and 37 introns in 14 genes often rearranged in cancer for base substitutions, indels, copy number alterations, and gene fusions. The average median sequencing depth was 1,154×.

Results. We observed 158 genomic alterations in 55 genes in 48 of 51 (94%) tumors (mean 3.1, range 0–9). The average number of potentially therapeutically relevant alterations was similar in primary (1.6, range 0–4) and in heavily pretreated metastatic cancers (2.0, range 0–4) ($p = .24$). The most common actionable alterations were in *PIK3CA* ($n = 9$,

phosphatidylinositol 3-kinase [PI3K]/mammalian target of rapamycin [mTOR] inhibitors), *NF1* ($n = 7$, PI3K/mTOR/mitogen-activated protein kinase inhibitors), v-akt murine thymoma viral oncogene homolog 1-3 ($n = 7$, PI3K/mTOR/AKT inhibitors), *BRCA1/2* ($n = 6$, poly[ADP-ribose] polymerase inhibitors), and *CCND1,2* and *CCNE* ($n = 8$)/cyclin dependent kinase (CDK)6 ($n = 1$) (CDK4/6 inhibitors), *KIT* ($n = 1$, imatinib/sunitinib), *ALK* ($n = 1$, crizotinib), *FGFR1,2* ($n = 5$, fibroblast growth factor receptor inhibitors), and *EGFR* ($n = 2$, epidermal growth factor receptor inhibitors). Our sequencing assay also correctly identified all six cases with *HER2* (*ERBB2*) amplification by fluorescence in situ hybridization when tumor content was adequate. In addition, two known activating *HER2* mutations were identified, both in unamplified cases.

Conclusion. Overall, 84% of cancers harbored at least one genomic alteration linked to potential treatment options. Systematic evaluation of the predictive value of these genomic alterations is critically important for further progress in this field. *The Oncologist* 2014;19:453–458

Implications for Practice: The technical ability to perform molecular profiling in the clinic is broadly available; it is now critically important to focus on assessing the clinical utility of molecular profiling as a patient selection tool.

INTRODUCTION

An increasing number of molecularly targeted drugs are available in the clinic as approved drugs (Table 1) or in the context of clinical trials (<http://www.clinicaltrials.gov>). These drugs target specific molecular abnormalities, including mutated protein kinases and amplified or rearranged genes. Cancers that carry these abnormalities often, but not always, respond to the corresponding targeted therapies. For example, the *HER2* gene-amplified breast cancers benefit from *HER2*-targeted therapies [1]. Chronic myeloid leukemia with the *BCR-ABL* translocation responds to inhibitors of the *BCR-ABL*

kinase [2]. Lung cancers with activating mutations of *EGFR* can benefit from epidermal growth factor receptor (EGFR) inhibitors [3], and lung cancers that carry an activating rearrangement of the *ALK* kinase often respond to anaplastic lymphoma kinase (ALK) inhibitors [4]. *BRAF*-mutant melanoma may respond to v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) inhibitors [5], and activating mutations in *c-KIT* or *PDGFR* render gastrointestinal stromal tumors sensitive to KIT inhibitors [6]. The repertoire of genomic abnormalities and their incidence differ across different histologic types of

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Table 1. U.S. Food and Drug Administration-approved molecularly targeted drugs for cancer

Drug	Primary molecular target(s)
Ado-trastuzumab emtansine	HER2
Afatinib	EGFR, HER2, HER4
Axitinib	PDGFR, VEGFR
Bevacizumab	VEGF
Bosutinib	BCR-ABL, SRC
Cabozantinib	MET, RET, VEGFR, KIT, FLT3, TIE2, TRKB, AXL
Cetuximab	EGFR
Crizotinib	ALK
Dabrafenib	BRAF
Dasatinib	BCR-ABL, SRC
Erlotinib	EGFR
Everolimus	mTOR
Gefitinib	EGFR
Imatinib	BCR-ABL, KIT, PDGFR
Lapatinib	HER2, EGFR
Nilotinib	BCR-ABL, PDGFR, KIT
Panitumumab	EGFR
Pazopanib	VEGFR, PDGFR, KIT
Pertuzumab	HER2
Ponatinib	BCR-ABL, VEGFR, FGFR, TIE2, FLT3
Regorafenib	VEGFR, RET, KIT, PDGFR, RAF
Sorafenib	RAF, VEGFR, PDGFR
Sunitinib	VEGFR, PDGFR, KIT, RET, FLT3
Temsirolimus	mTOR
Trametinib	MEK
Trastuzumab	HER2
Vandetanib	VEGFR, EGFR, RET
Vemurafenib	BRAF
Vismodegib	Smoothed (Hedgehog pathway)
Ziv-aflibercept	VEGF

Source: The website <http://www.fda.gov> was used to identify approved cancer drugs. Drug targets were extracted from the National Cancer Institute website <http://www.cancer.gov>.

cancer, but most abnormalities are not unique to any particular cancer type [7]. Although the same genomic abnormality may play a more important driver role in one type of cancer compared with another, there is also evidence to support that different types of cancers could respond to the same biologically targeted agent if they harbor the sensitizing genomic abnormality. For example, *HER2*-targeted therapies are effective in gastric and esophageal adenocarcinomas that have *HER2* gene amplification [8]. The BRAF inhibitor vemurafenib has shown promising results in patients with *BRAF* mutant metastatic papillary thyroid cancer and malignant histiocytosis [9]. The purpose of the current study is to survey the potentially targetable genomic abnormalities in primary and metastatic breast cancers using a standardized, commercially available next-generation sequencing (NGS)-based genomic profiling assay on routine clinical tissue samples.

Several studies have examined the mutational landscape of breast cancer using whole genome or partial genome sequencing [10–12]. Many of the initial whole genome and whole exome sequencing studies included very few patients and had limited sensitivity because of low sequencing depth. Other studies included a larger number of patients but restricted the analysis to a modest number of known oncogenic mutations [13–15]. The most comprehensive genomic analysis of breast cancer was recently reported by the Cancer Genome Atlas Network (TCGA) [16]. Whole exome sequencing was performed on 507 breast cancers with modest sequencing depth (30% of target sequences had coverage <20-fold). Low to moderate coverage limits the sensitivity to detect genomic events that may be restricted to relatively small tumor cell subpopulations. In aggregate, the above studies have established *TP53* and *PIKCA* as the most frequently mutated genes in breast cancer and also revealed a large number of low-frequency potentially druggable genomic anomalies in variable proportions of breast cancers. These observations raise the possibility that subsets of breast cancers may be candidates for targeted therapies aimed at rare genomic abnormalities. In this study, we performed full sequencing of 3,230 exons in 182 cancer-related genes and 37 introns in 14 genes that are frequently rearranged in cancers. As a result of the targeted sequencing method and the high, uniform coverage (>99% of exons covered $\geq 100\times$), our detection sensitivity for minor allele variants is significantly higher than those achieved in the TCGA. The analysis was performed using a standard operating procedure in a CLIA-certified (Clinical Laboratory Improvement Amendments, <http://www.cms.gov/clia>), CAP-accredited (College of American Pathologists) commercial molecular diagnostic laboratory; therefore, the results can be directly incorporated into clinical trials as patient selection criteria or used for medical decision making.

MATERIALS AND METHODS

Patients and Samples

Fifty-one breast cancers were analyzed, including primary tumor biopsies in 1 stage I, 17 stage II, and 15 stage III primary breast cancers and 18 total stage IV metastasis biopsies of 13 soft tissue, 3 liver, and 2 bone tumors. The mean age was 52 years (range 28–86 years), 58% ER+, 20% HER2+, 31% triple negative. Fine-needle aspirations (FNA) of the primary cancers were obtained prospectively at the time of diagnosis before any therapy in the context of a prospective biomarker discovery protocol at MD Anderson Cancer Center. Metastatic cancer biopsies, also obtained through FNA, were collected in the context of a prospective biomarker validation trial [17]. Patients with metastatic breast cancers had received at least two prior lines of therapy for metastatic cancer and were exposed to an average of seven different drugs (range 5–17), including adjuvant therapy before the biopsies were obtained. Both tissue collection studies were approved by the Institutional Review Committee of the University of Texas MD Anderson Cancer Center, and all patients signed informed consent. Analysis was performed on the already collected tissue samples, and the results were not included in the medical records or used for treatment decision making.

Figure 1. Schematic illustrating tumor sample preparation, sequencing library preparation, the analysis pipeline, and clinical reporting. Reprinted with modification from Nature Biotech 31:1023-1031, 2013.

Next-Generation Sequencing (NGS)-Based Genomic Profiling

Two FNA aspirates were pooled into a single vial for subsequent molecular analysis, and NGS-based targeted sequencing was performed by Foundation Medicine. Genomic DNA was extracted using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega, Madison, WI, <http://www.promega.com>) and quantified using a PicoGreen fluorescence assay (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). Fifty to 200 ng of DNA was sheared to 100–400 base pair fragments by sonication, followed by end-repair, dA-addition, and ligation of indexed, Illumina (San Diego, CA, <http://www.illumina.com>) sequencing adaptors. Sequencing libraries were hybridization captured using a pool of >24,000 individually synthesized 5'-biotinylated DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA, <http://www.idtdna.com/Home/Home.aspx>) that were designed to target 182 cancer-related genes and 37 additional introns in 14 genes often rearranged in cancer, corresponding to 1.14 million total base pairs (supplemental online Table 1). DNA sequencing is performed using the HiSeq-2000 instrument (Illumina) with 49 × 49 paired-end reads. Point mutations (base substitutions), short insertions/deletions (indels), focal amplifications, homozygous deletions, and chromosomal rearrangements were analyzed using Bayesian algorithms, local assembly, comparison with process-matched normal controls, and analysis of chimeric pairs, respectively. The method was optimized to detect >5% mutant allele frequency (MAF) of base substitutions and >10% MAF of indels with >99% accuracy. The validated accuracy of copy number alterations was >95%. Methodological details and analytical validity of the assay were reported previously [18]. The results were annotated and interpreted through dbSNP, COSMIC, and medical literature to assemble the final report of actionable genomic alterations. We did not analyze matched normal specimens; however, all reported mutations have been identified in previously published cancer-sequencing studies and were therefore considered somatic events. An alteration was categorized as potentially “actionable” if linked to an approved therapy in breast cancer or another solid tumor, or if it mapped to a pathway targetable with an approved drug or a drug in clinical trials.

RESULTS

All biopsies yielded the 50 ng of DNA required for analysis. The average median sequencing depth was 1,154-fold with >99% of nucleotides covered ≥100-fold. The time from DNA processing to final report required 7–14 days (Fig. 1). We

Figure 2. Pie chart of the alteration classes in the 51 breast cancers sequenced in this study.

observed 158 genomic alterations in 48 of 51 (94%) tumors (mean 3.1, range 0–9) in 55 genes. In total, 30 of 33 (91%) primary and 18 of 18 (100%) metastatic breast cancers had at least one genomic alteration. The 158 genomic alterations observed included 45 base substitutions (15%), 24 short insertions/deletions (28%), 63 focal amplifications (40%), 18 homozygous deletions (11%), and 8 rearrangements (5%) (Fig. 2). Overall, 84% of samples harbored at least one actionable alteration (Fig. 3). These actionable targets included the following: *PIK3CA* ($n = 9$, 18%), *HER2* ($n = 9$, 18%), *NF1* ($n = 7$, 14%), *MCL1* ($n = 6$, 12%), and *PTEN* ($n = 5$, 10%). Twenty-five other genes with actionable alterations were identified, occurring at lower frequencies ($n = 4$ or fewer, <10%).

The specific alterations that were observed in the most frequently actionable targets included the following: *PIK3CA* C420R, E542K, E545A, E545K, H1047R (H1047R occurred in four cases); *HER2* amplification and *HER2* S310F and L755S mutations; *NF1* truncation; *MCL1* amplification; *PTEN* truncation; and *PTEN* S170I mutation. Sequencing-based *HER2* copy number assessment correctly identified *HER2* gene amplification in all six cases that were *HER2*-positive by routine clinical testing and had adequate tumor cellularity for NGS analysis. Additional mutations in other well-known oncogenes were observed, including *AKT1* E17K (three cases); *ALK* V757M; *DNMT3A* R882C; *ESR1* Y537C, Y537N, and D538G; *KIT* K642E; and *KRAS* G12D. Numerous other alterations with no available therapeutic implications were also observed in each cancer, including *MYC* amplification ($n = 8$, 16%) and *TP53* alteration ($n = 32$, 63%: 31 mutations and 1 homozygous deletion), which were the most frequent alterations observed.

Thirty-nine (76%) tumors exhibited multiple alterations (Fig. 4). Only nine (18%) tumors exhibited single genetic alterations, and three (6%) tumors exhibited no detectable alterations. The average number of alterations was 2.9 in

Figure 3. Distribution of alterations observed in this study. For abbreviations and information on these genes, please see <http://www.genecards.org>.

Abbreviations: FGFR, fibroblast growth factor receptor; MEK, mitogen-activated protein kinase kinase; PARP, poly(ADP-ribose) polymerase; PI3, phosphatidylinositol 3; PI3K, phosphatidylinositol 3-kinase.

primary tumors and 3.4 in heavily pretreated metastatic cancers ($p = .31$). The average number of actionable alterations was 1.6 in primary (range 0–4) and 2.0 (range 0–4) in heavily pretreated metastatic cancers ($p = .24$).

DISCUSSION

This study, along with numerous other reports, demonstrates that comprehensive sequencing of potentially druggable genes can be performed on small, routine, diagnostic needle biopsies in a clinically relevant time frame. The incorporation of NGS to profile cancer biopsies for therapeutic targets has become a reality in the clinic. Our results also indicate that a large fraction of breast cancers contains genomic abnormalities that may render them susceptible to approved or investigational therapies. The distribution and frequency of the most frequent genomic alterations observed in this study are similar to the findings reported by the TCGA breast cancer project including *TP53* and *PI3KCA* mutations and *HER2* amplification [16]. Importantly, sequencing-based assessment of *HER2* gene copy number correctly identified six of the six clinically *HER2*-amplified cases when assay requirements were met. We also identified functional mutations in the *HER2* gene (S310F, L755S) in two cases in the absence of amplification. Both of these mutations have previously been reported in the literature; S310F is an activating mutation [19], and L755S is a mediator of resistance to lapatinib [20]. We also detected numerous, less frequent but directly targetable alterations in *KIT* (K642E, an activating mutation commonly seen in

gastrointestinal stromal tumors [21] and in acral and mucosal melanomas [22]), *ALK* (V757M, mutation in the extracellular domain that was previously observed in colorectal cancer [10]; *ALK* translocations cause non-small cell lung cancer, and mutations cause neuroblastoma [23]), and *AKT1* mutation (E17K, a constitutively activating mutation that was also seen in colorectal, endometrial, lung, and ovarian cancers and acute leukemias [24]). We observed amplifications of the *EGFR*, *FGFR1*, *FGFR2*, *AKT2*, and *AKT3* genes, all of which can be directly targeted by approved or investigational drugs.

A second collectively large group includes alterations that are not direct drug targets but represents biological pathways that could be targeted by drugs. These include deletion and truncation of neurofibromin/*NF1* (Y1625fs*5, A2646fs*14, Q1399*, W1559*, and c.1393-1G>T splice site mutation), a negative regulator of the *RAS* signal transduction pathway, and a mutation in *KRAS* (G12D, a mutation that confers resistance to anti-EGFR therapy in colorectal cancer). Amplification of *MCL1* gene that encodes BCL2-like protein inhibits apoptosis and amplifications in *cyclins E* and *D*, and *MYC*, as well as homozygous loss of *PTEN*, *BRC42*, and truncation of *BRC41* (S1253fs*10, Q1756fs*74, K1759fs*70) and *BRC42* (V1610fs*4, L2092fs*7).

It is also important to recognize that almost all cancers harbored multiple abnormalities. The average number of targetable alterations per sample was surprisingly similar, 1.6 in primary versus 2.0 in heavily treated metastatic breast cancers. The multiplicity of anomalies suggests that ultimately

Figure 4. Comprehensive annotation of genomic alterations of 51 breast cancers.

combinatorial therapies may be required for optimal efficacy. Studying the functional interaction between the multiple somatic alterations that tumors acquire and the equally large number of functional germline polymorphisms that we all carry represents a very important and fertile ground for research. However, it is also important to remember that, despite the multiplicity of functional genomic alterations, targeting single alterations can provide clinical benefit (e.g., antiestrogens, HER2-targeted therapies, etc.), which justifies clinical testing of targeted therapies.

The increasing clinical availability of NGS-based profiling assays and the results that they generate raise several important clinical questions. The most important one is how to act on the results. Currently, all U.S. Food and Drug Administration (FDA)-approved therapies that target a specific molecular abnormality in breast cancer are HER2-targeted agents (trastuzumab, lapatinib, pertuzumab, and ado-trastuzumab-emtansine). However, there are 26 other FDA-approved drugs (Table 1) that target specific somatic molecular abnormalities seen in cancer, most of which are encountered at low frequencies in breast cancer. It is attractive to consider the use of these targeted agents in tumor types in which they have not been approved to evaluate the predictive utility of target profiling [25, 26]. The safety profiles of already approved agents are already well established, and the key challenge is to assess clinical benefit. Many academic institutions pursue “molecular triaging” or “basket” studies that involve performing molecular target profiling and using the results to steer patients to clinical trials that test targeted therapies [27]. The U.S. National Cancer Institute recently announced plans for the MATCH trial that

aims to provide access to therapies that target the specific mutations found in a cancer [28]. Pharmaceutical companies are also considering providing a portfolio of their approved drugs and some investigational agents for molecular triaging studies that encompass multiple cancer types; however, no such clinical trial is open yet. The fragmentation of the patient population into very small, molecularly defined subsets will be a challenge during the implementation of these trials. It is hard to envisage efficiently running separate studies for *BRAF* mutant, *HER2* mutant, *AKT* mutant, *ALK* mutant, *cKIT* mutant, and *FGFR*-amplified breast cancer subsets. More importantly, only a minority of cancer patients receive their care through large academic centers and have access to these studies; overall, only 5%–10% of U.S. cancer patients participate in clinical trials [29].

One could also consider other innovative ways to harness the broad scientific interest in this field and the motivation of patients with incurable diseases to broaden their treatment options. Establishment of a nationwide registry of molecularly targeted therapies of rare genomic abnormalities could rapidly move the field forward. The registry would collect basic clinical and molecular information and simple, but informative, outcome data such as duration of therapy (i.e., a composite endpoint of tolerability and cancer control). Such registry could even be linked to coverage for treatment and maintained by the Center for Medicaid and Medicare Services or other third-party payers. Drug activity could be assessed and made public at predefined milestones, for example, after accruing the first 100 patients nationwide with a particular molecular anomaly. The registry would not replace rigorous clinical trials but could identify clinical scenarios in which further trials are needed.

CONCLUSION

In summary, NGS-based genomic profiling of DNA from breast cancer needle biopsies to assess potential therapeutic targets is readily available. Target profiling showed a high frequency of genomic alterations linked to potential treatment option with approved or investigational drugs. These alterations include multiple different types of abnormalities, including gene amplification and deletions, frame shifts, small insertions and deletions (indels), single-nucleotide substitutions, and gene rearrangements with fusion genes. These results raise a broad spectrum of distinct clinically testable therapeutic hypotheses for individual patients. Testing the clinical efficacy of these treatment options represents a major challenge for the traditional clinical trial system as a result of the varied and individually small patient subsets. Innovative approaches to provide access to potentially effective drugs and to capture systematically the outcome of therapy are needed to move the field forward and to provide benefit to the greatest number of patients without long delays.

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DISCLOSURES

Lajos Pusztai: Foundation Medicine (RF); **Roman Yelensky:** Foundation Medicine (E, IP, OI); **Kai Wang:** Foundation Medicine (E, OI); **Gary Palmer:** Foundation Medicine (E, OI); **Philip Stephens:** Foundation Medicine (E, OI); **Maureen Cronin:** Celgene (E); Celgene, Foundation Medicine (OI); **Vincent Miller:** Foundation Medicine (E, OI). The other authors indicated no financial relationships.

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