Incorporating Genomics into Breast Cancer Clinical Trials and Care

Adel Tabchy, Cynthia X. Ma, Ron Bose, and Matthew J. Ellis

Abstract
Advances in DNA sequencing provide the potential for clinical assays that are timely and affordable and use small amounts of clinical material. The hypothesis has therefore been raised that marked improvements in patient outcomes will result when DNA diagnostics are matched to an armamentarium of targeted agents. While this may be partially true, much of the novel biology uncovered by recent sequencing analysis is poorly understood and not druggable with existing agents. Significant other challenges remain before these technologies can be successfully implemented in the clinic, including the predictive accuracy of pathway-based models, distinguishing drivers from passenger mutations, development of rational combinations, addressing genomic heterogeneity, and molecular evolution/resistance mechanisms. Developments in regulatory science will also need to proceed in parallel to scientific advances so that targeted treatment approaches can be delivered to small subsets of patients with defined biology and drug reimbursement is available for individuals whose tumor carries a mutation that has been successfully targeted in another malignancy, as long as they agree to participate in an outcome registry. Clin Cancer Res; 19(23); 6371–9. ©2013 AACR.

Introduction
The first instance of successful oncogene targeting began with the discovery of HER2 (ERBB2) amplification in breast cancer (1). Testing for this somatic gene rearrangement effectively targeted by the monoclonal antibody trastuzumab led to a significant improvement in patient survival (2). Somewhat ironically, since then, no other gene mutation or gene rearrangement drug pairs in breast cancer have been approved, in contrast with other diseases such as nonsmall cell lung cancer, where several targets are now routinely the focus of therapy. In general, progress has been slow (3). Recently, advances in high-throughput sequencing technologies allowed a comprehensive characterization of genetic aberrations that underlie breast cancer in individual patients, promising an increase in the pace of therapeutic advances for the disease (4–6). This review assesses prospects for these advances in the near term and considers barriers and solutions to clinical research and implementation. Some of the barriers that must be methodically addressed include high-throughput functional and pharmacologic characterization of genomic alterations, computational tools for data interpretation and target identification, an understanding of the clinical implications of tumor heterogeneity and resistance mechanisms, the cost of care, reimbursement, clinical trial design in small populations, and regulatory challenges (7, 8).

Genome-forward trial design
A major source of the optimism that DNA-based diagnostics will be implemented is the availability of hundreds of drugs that have been specifically developed to target the products of genes in cancer-related pathways. The convergence of predictive biomarker science, of which sequencing technologies is only one aspect, with the unprecedented growth in drug development, is therefore initiating a new generation of clinical trials in which biologic and genomic considerations are not an afterthought, conducted in a retrospective and often underpowered way (“genome reverse”), but prospectively addressed through hypothesis and genome-driven trials in biomarker-defined populations (“genome forward”). The biology/biomarker/hypothesis first approach clearly requires a great deal of rigor, particularly in biomarker development, but the hope is that by meeting the diagnostic challenge large improvements in smaller clinical trials will be achieved, which is of course the opposite of the usual experience of small improvements in large clinical trials when drugs are studied in patients with undefined biology. The litany of therapeutic possibilities raised by The Cancer Genome Atlas (TCGA), as well as other large-scale sequence efforts, has recently been reviewed (4). This article provides an opportunity to further review these data and to discuss some of the barriers to clinical translation.
Massive Parallel Sequencing—Transition from Research to Clinical Testing

The documentation of the first human genome by Sanger sequencing, reported in 1991, required a decade of work by many laboratories and cost over $2 billion. In 2005, next-generation or massive parallel sequencing (MPS) emerged, allowing faster and more sensitive analysis of the cancer genome. This improvement in sequencing technology promises extensive information on somatic mutations present in individual cancers with a clinically useful turnaround time and at reasonable cost. In the research setting, a whole genome can be sequenced in about a week or less at a cost of a few thousand dollars. Alternatively, selected hotspots where mutations occur at high frequency in cancer can be sequenced in a few days for a few hundred dollars. However, costs relating to the clinical laboratory setting can inflate these estimates considerably. Whole-genome sequencing (WGS) allows the unbiased identification of all classes of somatic variation, including copy number aberrations, DNA rearrangements (translocations and inversions) as well as point mutations and small insertions and deletions (indels) at approximately 30 to 60 mean depth of coverage of the tumor and paired normal (germline) DNA (9). Whole-exome sequencing, on the other hand, allows detection of point mutations and small insertions/deletions in coding sequence but not larger-scale DNA rearrangements. High-throughput genotyping platforms (multiplexed screens) involving several technologies now permit the evaluation of heterogeneous cancer specimens at sensitivities higher than those with the Sanger approach (10); however, there is a requirement to predefine a set of candidate mutations so these panels are not an unbiased discovery approach. MPS can also be used to interrogate RNA and the epigenome (methylated DNA patterns) with varying depth and breadth depending on the application and goals of analysis. RNA-seq (transcriptome sequencing) determines the nucleotide sequence of cDNA derived from mRNA, microRNA, and other RNAs; it can therefore detect gene expression, gene fusion events, and alternative splice isoforms. Unfortunately, the reverse-transcriptase step (used to convert RNA to cDNA) is too error prone for RNAseq to identify point mutations reliably, but RNAseq has great value to determine the expression of a mutant detected by DNA sequencing, which is one of the criteria for druggability.

Pharmacologic Annotation of Cancer Genomes

Our knowledge of the function of the majority of the mutations in breast cancer remains sketchy, and, because many mutations do not yet have a matched pharmacology, one might question whether we need extensive sequencing information when we do not know the clinical utility of much of the data. For today’s regulatory setting, we are still focused on the one drug, one gene, and one organ site model. This research paradigm therefore requires information on one drug-matched gene in hundreds of patients, not hundreds of genes on one patient. Thus, a reversal of the usual design of the MPS assay can be considered such that for each sequence run, many patient samples can be analyzed for the gene of interest using sample bar codes to distinguish one individual from another. This efficient process is very suitable for cheaply screening a population for mutations in a gene that occurs in perhaps one patient in fifty but is nonetheless important because a highly effective inhibitor is available. After all, breast cancer is so common that a mutation that occurs in just 1% of patients involves thousands of individuals and rivals the incidence of the rare leukemias for which great strides have been made with targeted therapy. Even mutations with a less than 1% frequency might be significant if studies are developed for a mutation class that occurs across organ sites such that in sum a viable trial population can be identified for a so-called “basket” clinical study. The tension among gene number, clinical utility, turnaround time, reimbursement, and cost will play out over the coming years as the actionable mutation list lengthens from the current rather short list of genome-forward possibilities (Table 1).

Therapeutic Insights from Large-Scale Genomic Efforts in Breast Cancer

Pioneering work more than 10 years ago demonstrated that breast cancer is a spectrum of diseases at a molecular level and can be classified into four main subgroups on the basis of mRNA expression profiling: luminal A, luminal B, HER2-enriched, and basal-like (11). More recently, a 50-gene model called the PAM50 was developed to segregate individual tumors in the research setting into these subtypes using single sample predictor methodology (12). The PAM50 was used as one of the organizing principles for the TCGA to report extensive data on breast tumors by DNA copy number, gene expression analysis, DNA methylation patterns, microRNA sequencing, and reverse phase proteins arrays. Consistent with the conclusion that the intrinsic subtypes should be viewed as different diseases, large differences in the "omics" data sets were observed within each intrinsic subtype. Furthermore, the frequencies of individual actionable aberrations were strongly influenced by intrinsic subtype (13).

Luminal type breast cancer

PIK3CA mutation is the most common significantly mutated gene in estrogen-receptor positive (ER+) luminal breast cancer, is an exploitable therapeutic target because these mutations are gain-of-function. Clinical proof-of-principle exists for targeting the PI3 kinase (PI3K) pathway in ER+ breast cancer since an inhibitor of mTOR (everolimus), a pathway component downstream of PI33CA, has been combined with exemestane to produce an improvement in progression-free survival in patients with advanced disease (4). However, in this study PIK3CA mutation status failed to predict benefit, pointing to a need for deeper discovery work on predictive biomarkers for rapamycin analogue therapy (14). These data also underscore a need to develop agents that directly target mutant PIK3CA to improve efficacy and
<table>
<thead>
<tr>
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<th>ClinicalTrial.gov</th>
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<tbody>
<tr>
<td>BYL719 (alpha specific inhibitor of PI3K)</td>
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<td>PIK3CA mutant</td>
<td>ER’+ advanced BC</td>
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<tr>
<td>MK-2206 (AKT inhibitor)</td>
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<td>BKM120 (pan-PI3K inhibitor)</td>
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<td>ER’ HER2’ advanced or metastatic BC refractor to aromatase inhibitor</td>
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<tr>
<td>FGF4-2100 (pan-PI3K and mTOR inhibitor)</td>
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<td>All approved agents</td>
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<tr>
<td>HER2 directed antibodies and HER2 kinase inhibitors</td>
<td>All approved agents</td>
<td>HER2 amplification</td>
<td>HER2’ breast cancer</td>
<td>Trastuzumab, pertuzumab, and lapatinib</td>
<td>All approved agents</td>
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<tr>
<td>FGFR small-molecule inhibitors and antibodies</td>
<td>N/A</td>
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<td>MK-2206</td>
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<td>CDK4/6 inhibitors</td>
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<td>HER2 mutation</td>
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<td>Neratinib</td>
<td>Phase II</td>
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</tbody>
</table>

NOTE: This table summarizes clinical trials of new targeted therapies in breast cancer. Not all of these studies require a genomic abnormality for eligibility and therefore cannot be classified as genome forward, but all have embedded genomic analysis to determine the sensitive population.

Abbreviations: BC, breast cancer; ER’, estrogen receptor positive.
tolerability over rapamycin analogues. In contrast with PI3CA, most of the significantly mutated genes are in known or novel (often putative) tumor suppressor genes, with the most common, not surprisingly being TP53. While TP53 mutation is not currently a targetable event, mutations in TP53 were significantly enriched in luminal B tumors and in tumors with a higher histologic grade (4, 13, 15–19), suggesting a potential role as a prognostic marker. For luminal A tumors, MAP3K1 is the most common mutated tumor suppressor. While for luminal A tumors endocrine therapy is the principal treatment option, a loss-of-function in the MAP3K1 stress response gene could theoretically lead to tumor persistence and late relapse (19). Another interesting significantly mutated gene is MLL3. This histone trimethyltransferase (4, 13, 15–19) also does not have a clear therapeutic strategy but it is interesting to note that randomized phase II data for the histone deacetylase inhibitor and epigenetic modulator entinostat showed activity in ER+ advanced breast cancer in combination with the aromatase inhibitor exemestane (4), raising the hypothesis that interactions between epigenetic modifiers and mutations in genes that affect the epigenome may be a rich area for predictive marker discovery.

As with other common solid malignancies, the gene amplification landscape provides most of the high frequent gain-of-function targets, including the TP53 antagonist MDM2 that is associated with luminal B status and therefore endocrine therapy resistance. Inhibitors of MDM2 are in phase I testing. CDK4 and CDK6 form a complex with cyclin D1 that is amplified in 40% of luminal breast cancer; phase II clinical trials of CDK4/6 inhibitors in combination with letrozole have completed phase II randomized clinical trial testing with positive outcomes (4, 13, 15–19). Finally, amplification of FGFR1 is a relatively common event in luminal breast cancer, and a spectrum of antibodies and small-molecule inhibitors are also in clinical development. Thus, the list of drugs that target specific somatic mutations classes in luminal-type breast cancer is beginning to lengthen (Table 1).

**Basal-like breast cancer**

Luminal-type and basal-like breast cancers are completely different diseases at the molecular level (4, 13, 15–19). Basal-like breast cancers show more similarity with other high-grade epithelial tumors such as squamous cell carcinoma of the lung or head and neck, as well as high-grade serous ovarian cancer and serous endometrial cancers. These are all platinum-sensitive diseases with high rates of TP53 mutation. Studies based on ovarian cancer–like platinum/taxane combinations in basal-like breast cancer are clearly a priority (4). The presence of 20% BRCA1 or BRCA2 mutations (germline and somatic) in basal-like breast cancers is another mechanistic link to serous ovarian cancer, and clinical trials testing PARP inhibitors have been activated (4, 13, 15–19). A strong genomic and proteomic signal for PI3K pathway activation exists for basal-like breast cancer. As opposed to ER+/luminal breast cancer, this occurs less from a mutation in the PIK3CA gene (9%) and more from a wide variety of mutations, deletions, or loss of negative regulators such as INPP4B or PTEN (4, 13, 15–19). The interest in potent broad-spectrum PI3K inhibition in triple-negative breast cancer (TNBC)/basal-like breast cancer is therefore growing (20).

**HER2-enriched breast cancer**

Clinical HER2-positive disease can be divided into at least two groups. One has high levels of HER2 protein phosphorylation, tends to be ER−, and is assigned to the HER2-enriched category by PAM50. The non–HER2-enriched HER2+ tends to be ER+/luminal and have a lower level of DNA amplification and pathway signaling (21). The efficacy of trastuzumab-based chemotherapy may be reduced in the luminal HER2+ group, and the HER2-E group may be hypersensitive to anthracyclines (22).

**HER2 mutation as an example of a new actionable target arising from next gene sequencing studies**

The potential of cancer genome sequencing to identify new therapeutic avenues is illustrated by the finding of HER2 mutations in tumor samples defined as “HER2−” by routine testing. These mutations are quite rare in primary disease, the incidence is between 1% and 2% in primary breast cancer, and their recognition was clearly greatly assisted by the TCGA effort (23). Although these mutations are rare overall, there may be patient populations that are enriched for these mutations. For example, a recent sequencing study of relapsed/metastatic lobular breast cancers found HER2 mutations in 23% (24). To date, 25 examples of somatic mutations in HER2 have been identified and tested in the laboratory for transforming properties and in HER2-targeted drug screens (Fig. 1A). Mutations were found clustered either in the tyrosine kinase domain (68% of cases) or on a dimerization surface of the extracellular domain (20% of cases). Over half of the mutations led to activation of HER2 in standard assays, and a quarter of cases had mutations that produced de novo resistance to lapatinib. For example, the expression of the HER2 780–781insGP mutation (abbreviated 780ins) in MCF10A cells produced colonies that had invasive and spiculated protrusions in Matrigel culture. The phenotype associated with this mutation was only partially inhibited with lapatinib but could be completely inhibited with the irreversible tyrosine kinase inhibitor, neratinib. Because all the HER2 mutations tested were sensitive to neratinib, but there were multiple examples of lapatinib-resistant mutations, a phase II clinical trial has been launched to screen for and use neratinib to treat patients with metastatic breast cancer with HER2 mutations (Fig. 1B). If this clinical trial is successful, it will expand the number of patients who benefit from anti-HER2–targeted therapies and it will be the first example of a sequencing-based diagnostic tool for the routine evaluation of breast cancer patients, at least in the advanced disease setting. Some of the more generalizable conclusions that we drew from the HER2 experience include the observation that a thorough understanding of the molecular pharmacology of a new
mutation class is an essential prerequisite for clinical trial design. Furthermore, assumptions regarding the frequency of a mutation class, based on primary treatment-naive samples, may not be valid when considering advanced disease. If a mutation is a driver of poor outcome it will be enriched in patients with stage IV disease and its significance greater than that suggested by its prevalence in early-stage samples. Druggable genome programs identify a fairly large number of additional tyrosine and serine threonine kinase mutations that occur at a low frequency at the individual gene level but as a class could be present in perhaps 20% of breast cancer (excluding PIK3CA; ref. 25). Clearly, the place to start is with functional and pharmacologic studies on these mutations, combined with RNAseq analysis to confirm expression, as a prerequisite to clinical trials. Treating with available drugs based on DNA sequence data alone may be a low-yield strategy if the pharmacology is not correct or expression from the mutation was not confirmed.

Genome Forward Studies in the Neoadjuvant Setting for Luminal-type Breast Cancer

A detailed overview of the coupling of biomarker and drug development in the neoadjuvant setting and triple-
negative breast cancer is presented discussed elsewhere in this CCR Focus section (21, 26). One population not commonly studied in the neoadjuvant setting are patients with clinical stage II or III luminal-type breast cancer. Despite often relatively slow growth, luminal tumors can still present with larger tumors and positive nodes, often in a mammographically dense breast, in association with lobular carcinoma or in the absence of screening. Neoadjuvant endocrine therapy has become an acceptable practice standard, with a number of studies showing a very clear improvement in breast conservation rates as significant as that for chemotherapy (27). Through the Mayo phase II consortium, and with support from Susan G. Komen for the Cure, a genome forward study is currently active in which a patient with ER+ HER2– breast cancer (BC) are biopsied at baseline for genomic evaluation (genomic evaluation phase). In this case, tumor DNA is extracted for PIK3CA gene sequencing (in future studies a panel of mutation matched therapies could be offered). Patients are started on standard neoadjuvant endocrine therapy for 28 days while waiting for the results of genomic analysis. Those with PIK3CA mutant tumors will be eligible to enroll in the AKT inhibitor trial (NCT01776008: A phase II study of neoadjuvant MK-2206 in combination with anastrozole in PIK3CA mutant ER+ HER2+ clinical stage II or III breast cancer) and those with PIK3CA wild-type tumors will be eligible to enroll in the CDK4/6 inhibitor trial (NCT01723774: A phase II study of neoadjuvant PD0332991 in combination with anastrozole in ER+ HER2+ clinical stage II or III breast cancer). Repeat tumor biopsies will be taken prior to the start of either MK-2206 or PD-0332991 on cycle 1 day 1 (C1D1) and after 2 weeks of combination therapy (C1D15) for the evaluation of early response biomarkers (early response biomarker phase), in this case the Ki67. Those with resistant tumors (Ki67 >10%) will go off therapy early to avoid futile treatment. Those with Ki67-responsive tumors (Ki67 <10%) will receive a total of 4 months of study drug therapy followed by definitive breast surgery to evaluate pathologic response (efficacy evaluation phase). If the tumor is exhibiting a biomarker response, there is an efficacy evaluation phase whereby the investigation will move on to examine other agents. In the future, one can envisage an expansion of this approach whereby standard neoadjuvant endocrine therapy is initiated while more extensive genomic analysis is undertaken (genomic triage phase). This would allow a wider spectrum of experimental agents to be added depending on each tumor genomic profile. An appealing aspect of the study is an early response evaluation phase in which a biopsy is taken to ensure the tumor has undergone growth arrest in response to treatment as this prevents patients from continuing treatment with an ineffective approach. If the tumor is exhibiting a biomarker response, there is an efficacy evaluation phase whereby the patient is treated with the experimental combination for a number of months to observe a path CR at surgery (Fig. 2).

**Genome Forward Studies in the Advanced Disease Setting**

Experimental drug monotherapy or experimental combinations for clean proof-of-principle is a much more practical proposition in the advanced disease setting than the neoadjuvant setting because patients have moved beyond the curable stage and thus riskier investigations are more justifiable (28). However, the accrual of an adequate number of specimens for diagnostic analysis is considered a challenge. Nonetheless, a multi-institutional prospective study in...
France funded by the Institut National du Cancer stratified patients with metastatic breast cancer to experimental or approved targeted agents matched to molecular aberrations identified by genomic sequencing (29). This study clearly showed that detailed prospective molecular analysis of advanced breast cancer was feasible, but relatively few patients had immediate access to a targeted agent, underscoring the need for more molecular pharmacology and biologic studies and more partnerships with pharmaceutical companies to increase the yield for patients for invasive biopsy procedures that may cause complications.

**Extreme responder analysis**

An investigational approach that is underutilized in breast cancer is the genomic analysis of patients who experience unusually extreme responses to a targeted agent. This approach is exemplified by a recent study in bladder cancer in which a patient responded unusually well to the rapamycin analogue everolimus. Genome sequencing revealed a loss-of-function mutation in TSC1 (tuberous sclerosis complex 1), a regulator of the mTOR pathway. Targeted sequencing revealed TSC1 mutations in about 8% of 109 additional bladder cancers examined, and TSC1 mutation was correlated with everolimus sensitivity (30). However TSC1 is not frequently mutated in primary breast cancer, and most of the samples analyzed in the Bolero 2 study were from primary tumors rather than from metastatic disease. Because MPS studies have clearly demonstrated that ER+ disease shows evidence of genomic progression upon relapse (31), it has become ever more important to accrue advanced disease specimens to address interactions between mutations and drug efficacy that will be missed if we do not have the relevant sample.

**Tumor heterogeneity and molecular evolution**

MPS has clearly shown that many breast cancers show evidence for multiclonoality, i.e., often mutations have low allelic frequencies, implying they are only present in a subpopulation. Even conventional histology has demonstrated extensive heterogeneity in common epithelial malignancies, exemplified in breast cancer by geographical separation of subclones of tumor cells harboring different patterns of HER2 overexpression by immunohistochemistry (32). Studies have shown that subclones in the metastatic sites are preexistent at the primary site, with low frequency subclones in the primary site being enriched in the metastatic site (33, 34). Also, patients with heterogeneity of hormone receptors and HER2 status between primary and metastatic sites have been shown to have a significantly worse outcome (35). Biopsying a single metastatic site may underestimate the molecular aberrations driving the cancer in a patient, and more intensive biopsies may not be feasible. Furthermore, it is unclear if such an aggressive biopsy schedule would actually improve patient outcomes. Clearly, we are entering an era in which ongoing molecular monitoring will be essential but repeated biopsy of disease within internal organs is an impractical proposition. The advent of analytic approaches that can detect circulating plasma tumor DNA may therefore be part of the future for the diagnosis and monitoring of advanced disease. The so-called "liquid biopsy" approach employs MPS to detect cancer-associated mutations in the peripheral blood. Sensitive platforms based on detection of mutation in circulating tumor DNA have demonstrated accuracy in showing genetic abnormalities present in tumor biopsies from primary and metastatic breast tumors (36–38). Circulating tumor DNA can clearly differentiate between patients with breast cancer and healthy controls, and therefore this technology has the potential to be used for diagnosis and for monitoring of patients in the metastatic setting (36).

**Pathway analysis versus mutation analysis**

Currently, there is a natural emphasis on the single gene, single-drug paradigm but clearly the cure of epithelial malignancies will ultimately require a more sophisticated approach, whereby all the relevant biologic parameters in a tumor are assessed as prelude to multidrug targeting. Increasingly sophisticated cancer informatics programs have been developed that place genomic data into the context of well-curated signaling and functional pathways to create networks, "interactomes" and "activitomes." While specific biologic processes can then be linked to clinical phenotypes, the programs require multiple examples of each class for statistical inference, i.e., they have not reached the point of diagnosing a druggable pathway activation event in an individual patient. Furthermore, because the actual biochemistry is inferred, but not directly observed, the hypotheses raised by these programs must be confirmed by directly observed biochemistry (39). Proteomic technologies, based on mass spectrometry or reverse phase protein arrays, clearly represent a promising approach in this regard. Individualized and integrated analysis of DNA, RNA, and protein/posttranslational modifications is the next technical challenge in cancer diagnostics.

**Conclusions**

Genome-forward clinical trial designs are clearly a challenge and require a great deal of preparatory work to secure both the hypothesis and the biomarker. Trials with these designs will require early communication with the U.S. Food and Drug Administration, especially the Center for Devices and Radiological Health so that the device in the trial (i.e., the sequencing approach) can be evaluated/regulated. However, even in the setting of a "standard" clinical trial in unselected populations, it makes very little sense not to make the effort to prospectively accrue tumor and germline DNA from every patient in every study. Allowing expensive drugs to be approved on the basis of a modest benefit with no samples to discover the sensitive and resistant populations can clearly now be seen as unethical and unsustainable. Solutions to ethical and technical barriers for universal sample accrual are clearly in hand, and we should embrace these
approaches as the cornerstone of clinical investigation in cancer.

Disclosure of Potential Conflicts of Interest

R. Bose has received honoraria from the speakers’ bureau from Genentech and RGA International. M.J. Ellis has ownership interest (including patents) in BioClassifier LLC. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: M.J. Ellis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Ellis

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Tabchy, R. Bose, M.J. Ellis

Writing, review, and/or revision of the manuscript: A. Tabchy, C.X. Ma, R. Bose, M.J. Ellis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Tabchy, M.J. Ellis

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