

Gene signature-guided Dasatinib therapy in metastatic breast cancer

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Translational relevance

This study is the first prospective clinical trial in breast cancer to test the clinical utility of drug specific predictive gene signatures. The trial demonstrates the feasibility of complex gene expression-based treatment selection and shows how separate markers can be prospectively assess in a single trial. Unfortunately, none of the 3 previously reported predictors validated in this prospective trial which probably reflects the more complex biology of tumors compared to in vitro models. Predictors derived from experimental models involving relatively homogeneous cell lines may only partially capture the broad range of cellular and organism level resistance mechanisms.

ABSTRACT

Purpose: Dasatinib has limited single-agent activity in unselected patients with metastatic breast cancer. Several gene signatures predictive of dasatinib response in vitro have been reported. The purpose of this 3-arm, Phase II study was to prospectively assess the utility of 3 previously-published gene signatures to select patients with clinical benefit from dasatinib.

Experimental Design: Patients with metastatic breast cancer underwent biopsy for gene expression profiling in an academic CLIA laboratory; those who were positive for any one of 3 predictive gene signatures (dasatinib sensitivity signature, SRC pathway activity signature and dasatinib target index) received dasatinib 100 mg po daily. The three marker-defined cohorts were analyzed separately, using early stopping rules for futility.

Results: Ninety-seven patients were enrolled, 93 underwent biopsy and 80% of the biopsies were sufficient for molecular testing. Thirty patients were positive for at least one signature and received treatment. Only 1 patient experienced clinical benefit and had stable disease over 300 days. All 3 arms were closed early for futility. There was one serious biopsy-related adverse event (hematoma and pain following a liver biopsy). There were no unexpected toxicities from dasatinib.

Conclusion: None of the three predictive gene signatures, although supported by preclinical evidence, defined tumors clinically sensitive to dasatinib as a single agent.

Introduction

Dasatinib is an oral tyrosine kinase inhibitor that inhibits several protein kinases including BCR-ABL, SRC, LCK, YES, FYN, c-KIT, EPHA2 and PDGFR-beta and also binds to several other kinases that are frequently expressed in tumor cells (1, 2). Many of the key targets of dasatinib, particularly SRC, are involved in the regulation of cell proliferation, survival and metastatic ability of cancer cells (3,4,5). Dasatinib is approved for the treatment of patients with all phases of chronic myeloid leukemia (CML) or Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL).

Two Phase II trials tested the efficacy and safety of single agent Dasatinib in metastatic breast cancer. One study included patients with triple-negative disease and the other, patients with hormone receptor or Her-2-positive cancers. Both studies showed limited anti-tumor activity, with objective response rates of 4.7% and 5.6%, respectively (6,7).

Several multivariate gene signatures have been proposed as predictors of dasatinib sensitivity based on preclinical data. Investigators identified 161 probe sets that were differentially expressed between dasatinib-sensitive and -resistant cell lines (n=22) and combined these into a gene signature that correctly predicted dasatinib sensitivity in 11 of 12 independent cell lines (8); this signature is referred to as the “cell line-derived dasatinib sensitivity signature”. Another proposed predictive signature included 73 probe sets that were up-regulated after transfection of SRC oncogene into breast epithelial cells and is believed to reflect SRC pathway activity; this signature predicted response to SRC inhibitors in vitro (9); this is referred to as the “SRC pathway index”. A third predictive signature was proposed based on known kinase targets of dasatinib, with gene expression weighted by the in vitro binding affinity (K_{d50}) of dasatinib

for that respective target; this is referred to as the “dasatinib target index” (DTI). This target index was also predictive of dasatinib sensitivity in cell lines (10). Remarkably, these three putatively-predictive gene signatures share few overlapping probe sets and represent three conceptually different development strategies. In the current study, we prospectively tested the value of these gene signatures to select patients with tumors which might be particularly sensitive to dasatinib therapy. Our hypothesis was that some, or all, of the three previously published predictive signatures will identify a patient population who benefits from single agent dasatinib therapy, defined as a clinical benefit (CB) rate of $\geq 25\%$.

Materials and Methods

Patients and treatment

Patients with metastatic breast cancer were eligible for the study if they had measurable disease by RECIST and had safely biopsiable metastatic lesion judged by an interventional radiologist. Both estrogen receptor (ER) positive and negative as well as HER2 positive and negative cancers were eligible. There were no limits on prior therapies but performance status had to be Zubrod scale ≤ 2 with acceptable organ functions (absolute neutrophil count $\geq 1,500/\mu\text{L}$, platelets $\geq 100,000/\mu\text{L}$, hemoglobin $\geq 9 \text{ gm/dL}$, total bilirubin $\leq 2 \times$ the upper limit of the normal [ULN], aspartate aminotransferase and alanine aminotransferase $\leq 2.5 \times$ ULN, serum creatinine $\leq 1.5 \times$ ULN and normal serum electrolytes). Key exclusion criteria included the presence of pleural or pericardial effusion, uncontrolled angina, congestive heart failure within 6 months, QT interval $> 450 \text{ msec}$, ventricular arrhythmia, gastrointestinal bleeding or diagnosis of

bleeding disorder. All patients signed a voluntary informed consent and underwent a metastatic tumor biopsy for gene expression analysis. Fine needle aspiration (FNA) was the preferred method of tissue sampling because of its safety and because it yields specimens enriched in neoplastic cells compared to core needle biopsies (11). Only patients whose tumor was predicted to be sensitive to dasatinib, based on any of the three gene signatures, received treatment. Those who were predicted to benefit received dasatinib 100 mg p.o. once daily until disease progression or unacceptable toxicity. Dose reductions for toxicity were as follows: For the 1st occurrence of \geq grade 3 toxicity, treatment was held until resolution to grade 1 and subsequently restarted at 70 mg. If grade 3 toxicity recurred, therapy was discontinued. For the 1st occurrence of clinically significant grade 2 toxicity, treatment was held until resolution of symptoms and was restarted without dose reduction. If grade 2 toxicity recurred dasatinib was reduced to 70 mg daily. Clinical and laboratory evaluation was repeated every 4 weeks during therapy and tumor response was assessed every 8 weeks by RECIST. This study was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center and is registered at www.clinicaltrials.gov as NCT00780676.

Statistical Design and Analysis

The trial followed a parallel, 3-arm, multi-step, Phase II design as described previously (12). Each marker arm (cell line derived dasatinib sensitivity signature, SRC pathway index, dasatinib target index) was considered as a separate study with its own early stopping rule for futility. Sample size calculations followed a parallel, multi-arm, multi-step, Phase II design with early stopping rules for futility if clinical benefit (CB) rate had a $\leq 7.5\%$ chance to be $\geq 25\%$, a

threshold selected because drugs with 25% benefit rate are commonly used in the treatment of metastatic cancer. CB was defined as partial response, complete response or stable disease for ≥ 6 months. Each marker-defined treatment arm was stopped for futility if 0 of 9 patients or ≤ 2 of 20 patients had CB. Under this stopping rule, the probability of early termination was 80% if the true CB rate was 10%, but was 7.5% if the true CB rate was 30%. The maximum size of each arm was 40. If the true CB rate was 25% in a sample of $n=40$, the 90% confidence interval for the observed CB rate was 16% to 38%. Therefore, a marker-drug combination arm would have been recommended for further study if the observed CB rate was $\geq 16\%$ in a given marker arm.

Biopsies and Gene Signatures

Four fine needle aspirations (FNA) from the most safely accessible metastatic lesion were obtained by an interventional radiologist using a 24-gauge biopsy needle following standard clinical procedures with or without image guidance at the discretion of the physician. One cytologic smear was prepared for Diff Quick staining and assessment of cellularity. Only samples with $\geq 50\%$ tumor cellularity were processed for molecular analysis. This threshold was established based on RNA dilution experiments which indicated robustness of cancer-derived gene signatures to up to 50% contamination with RNA from the host organ. Two FNA passes were pooled into one 1.5-ml RNAlater tissue protect tube (Life Technologies, USA) and were delivered at room temperature to the CLIA-certified molecular pathology laboratory at MDACC to be stored at 2-8 °C until gene expression profiling. RNA extraction (RNAeasy kit, Qiagen) and gene expression profiling were performed within 24-48 hours of obtaining the biopsies following a standard operating procedure compliant with CLIA guidelines. Gene expression

analysis was performed on the Affymetrix GSE 3000 DX Diagnostic System using Affymetrix U133A gene chips. The quality of the array hybridization was assessed the “simpleaffy” package in R v2.4.0, using scaling factor, percent present calls and 3'/5' signal ratios of housekeeping genes as QC metrics. All arrays were normalized using MAS5 with scale factor set to 1500. When multiple probe sets targeted the same gene, the probe set with the highest median intensity and greatest interquartile range was selected as the representative probe set. All gene expression values were log₂ transformed.

The dasatinib target index (DTI) was calculated as the sum of the gene expression values of the 19 kinase targets each divided by its corresponding log₂ transformed Kd₅₀ value as previously published (10,13) (supplemental table 1). We established the distribution of the DTI values in a reference cohort of 133 primary breast cancer FNAs processed identically and defined the highest 25% of target index values as "potentially drug sensitive" based on the assumption that the higher the DTI the greater the sensitivity to dasatinib (supplemental figure 1). The threshold was calculated over all 133 cases without separate thresholds for ER positive and negative cases. The DTI score corresponding to this percentile threshold was 3074; patients with DTI above this value were eligible for therapy in the DTI marker arm.

The SRC pathway activity score was derived from a published gene list which consisted of 73 genes from Affymetrix HG-U133Plus2 arrays that were induced by forced expression of SRC (9). Only 46 of these genes were represented on our array platform, we used the average expression of these 46 genes as the measure of the SRC pathway activity (supplemental table 2). We assessed the distribution of the SRC pathway activity score in our reference population of 133 breast cancers and designated the top 25% to indicate high SRC pathway activity, without defining separate thresholds by ER status (supplemental figure 10-11). This threshold

corresponds to a score of 2127 and patients with scores above this were eligible for treatment in the activated SRC Pathway marker arm.

A multi-gene dasatinib response predictor was derived from comparison of dasatinib sensitive and resistant cell lines using data from reference 8. To identify differentially expressed genes between dasatinib sensitive and resistant cases, two-sample unequal-variance t tests were performed on the gene expression profiling of the 23 breast cancer cell lines and the genes were ranked by p values and false discovery rate (FDR). The top 142 probe sets (FDR<0.1) were included in a predictor that was trained on the cell line data using a weighted voting algorithm and validated on an independent cell line data set (10) (supplemental table 3). A patient was eligible for treatment in this Cell Line Derived Sensitivity signature arm if the sensitivity index value was > 0.

All three prediction algorithms were locked and an automated script performed the calculations for study arm assignment (supplemental table 4, supplemental figure 2). The technical reproducibility of the predictors was examined in repeat profiling of the same RNA samples and in repeat FNA biopsies from the same tumor. We also examined consistency of results between matching core needle and FNA biopsies of the same tumor (supplemental table 5-10, supplemental figure 3-9). These results as well as further details of the development of these three multi-gene predictors for clinical testing use were published in reference 10 and are also summarized in the Supplementary Methods (Supplemental table 11-17, supplemental figure 12-14) . If an individual was "positive" for more than one predictor, the patient was assigned to the marker arm for which she had the highest percentile ranking (i.e. was the “most” positive for).

In this study no cell lines were used since tissue biopsies obtained from patients who were enrolled in the clinical trial. Information about authentication of cell lines that were used to identify predictor signatures has already been published and cited in this article (8, 9, 10).

Results

Biopsy success rates

Ninety seven patients were enrolled in the study, of whom 93 underwent biopsies for gene expression profiling between June 2009 and May 2012. Of those who did not undergo biopsy, 2 withdrew consent, 1 had insurance disapproval and 1 patient had low platelet count. Ninety-six biopsy sessions were performed, 1 patient had two sites biopsied and 2 patients underwent repeat biopsy due to initial inadequate sampling. There were 26 (27%) lymph node biopsies (13 axillary, 7 supraclavicular, 2 cervical, 1 internal mammary, 1 mediastinal, 1 infraclavicular, 1 retroperitoneal), 18 chest wall and 16 skin or superficial tumor involvement of soft tissue including breast. Eighteen patients had liver, 13 bone, 4 had lung or pleura and 2 had adrenal gland biopsies. Ten of the 96 biopsies were core needle biopsies and 1 was a skin punch biopsy based on the judgment of the treating physician or interventional radiologist. There was one biopsy related complication involving hematoma and pain after a liver biopsy that required brief hospitalization.

Fourteen biopsies (14%) had low cellularity and could not be processed for gene expression profiling, 5 additional biopsies (6% of all RNA extractions) failed array QC due to low RNA quantity ($< 0.8 \mu\text{g}$ total RNA) or quality (260/280 ratios outside of 1.8 to 2.1). For

specimens that passed QC, the median time from biopsy procedure to prediction results was 7 days (range 3-16). Among the 30 patients who tested positive for at least one gene signature, 7 were positive for two different signatures (5 were double positive for the DTI plus the Cell Line Derived Sensitivity signature and 1 patient each for DTI plus SRC pathway and SRC Pathway plus Cell Line Derived Sensitivity signature). Figure 1 is a CONSORT diagram showing patient disposition after accrual to the molecular screening phase of the study.

Efficacy and safety results

Thirty patients had positive gene signatures and met all other eligibility criteria for treatment. Thirty three percent of DTI scores were above the threshold for positivity, a slightly higher proportion than expected for a threshold that was set to represent the 75% percentile. Nineteen and 12% of SRC Pathway and the Cell Line Derived Sensitivity scores were above the threshold, which were lower than expected. Thirteen patients were treated in the DTI arm, 8 in the SRC pathway activity arm and 9 in the cell line derived response predictor arm. Patient characteristics for those who received treatment are presented in table 1. Only one patient had HER2-positive breast cancer and 9 (30%) had ER positive tumor; interestingly, 7 of the 9 ER-positive tumors were in the SRC Pathway arm. Twenty-nine patients had more than 3 prior lines of chemotherapies for metastatic breast cancer and all ER positive cancers had at least 3 prior lines of endocrine therapy.

No clinical benefit was observed in the SRC Pathway and Cell Line Derived Sensitivity signature arms. There was one patient in the DTI arm who had stable disease for over 300 days (7.7%). She was a 59 year old patient with ER-positive metastatic breast cancer involving her

bone and liver for 9 years who received multiple prior lines of endocrine therapy and chemotherapies (CMF, paclitaxel, tamoxifen, nab-paclitaxel, capecitabine, ixabepilone, and liposomal doxorubicin). Two additional patients had stable disease lasting 103 and 105 days, one each in DTI and Cell Line Derived Sensitivity signature arms, respectively. The median progression-free survivals were similar in all three arms: 61 days in the DTI arm (range 6 - 300), 46 days (range 17 - 80) in the SRC Pathway arm and 44 days (range 16 - 105) in the Cell Line Derived Sensitivity signature arm. All three arms closed early due to lack of activity. The DTI arm had passed the first interim futility analysis (1/9 CB rate) but due to slow accrual and low overall activity across all arms, this marker arm was also terminated after 13 patients were treated along with the SRC pathway arm (n=8) which has not quite reached the first interim futility mark. Initial accrual to the study was brisk, 59 patients (61%) were accrued in the first 12 months; however, accrual slowed as the limited antitumor activity and modest biopsy positivity rates became apparent; only 10 patients were accrued in the last 10 months before closing the trial.

There were no unexpected toxicities. Four patients had grade 3 fatigue, one patient had grade 3 pleural effusion, one patient had grade 3 anemia, one patient had grade 3 neutropenia, one patient had grade 3 headaches and one patient had grade 3 nausea that were considered possibly related to Dasatinib treatment.

Discussion

Many predictive gene signatures for various therapies have been developed from in vitro models but few were tested in prospective clinical trials to assess their predictive value (13, 14).

This study represents the first prospective clinical trial in breast cancer that uses patient enrichment design to test the clinical utility of cell-line derived predictive multi-gene signatures. The study demonstrates that metastatic biopsies can be safely obtained for molecular analysis in the context of clinical trials and gene expression profiling can be performed in an academic molecular pathology CLIA setting with rapid turnaround. The tissue distribution of biopsies reflects the primary concern for patient safety and is appropriately skewed towards superficial lymph node and soft tissues (62% of all biopsies). QC failure rates were close to 20% but most failures occurred at the biopsy level due to low cellularity (15%). Bone biopsies yielded particularly low success.

We examined three conceptually different predictive signatures for dasatinib sensitivity. The two previously published signatures derived entirely from cell line experiments had to be slightly modified in order to apply these to human gene expression data; these modifications were described in detail in a previous publication (10). All prediction scores were calculated on prospectively collected metastatic cancer biopsies. The distribution of score values indicated that our a priori defined thresholds from primary breast cancers appropriately identified the metastatic cases in the high end of the distribution curves; 67th, 81st and 88th percentiles for the DTI, SRC and Cell Line Sensitivity scores respectively. Despite selecting patients with the highest scores, there was no significant clinical activity in any marker arm.

Predictive gene signatures for individual drugs, despite promising observations in preclinical trials, have not yet proven their clinical value. Retrospective “validation” of drug-specific predictive signatures derived from experimental models applied to patient data tend to yield substantially lower accuracy than hoped for (14, 15, 16). This may reflect the more complex biology of tumors compared to traditional in vitro models. Within tumors cellular

heterogeneity is a major potential confounder that can weaken the predictive value of multi-gene predictors. Different subpopulations of neoplastic cells within the same cancer may have different levels of drug resistance mediated by different mechanisms (17). Predictors derived from the much simpler experimental models involving relatively homogeneous cell lines may only partially capture the broad range of cellular and organism level resistance mechanisms. This limitation applies not just to predictors but drugs as well. Many drugs that have shown impressive activity against breast cancer cells in vitro (EGF-inhibitors, proteasome inhibitors, farnesyltransferase inhibitors, etc.) failed to produce similar activity in clinical trials (18,19,20).

While cell-line derived, drug specific, predictive gene signatures have not yet established their value as patient selection tools, several single-gene markers emerged as clinically useful predictive markers. Specific mutations in BRAF, EGFR, RAS, amplification of the HER2 gene and translocations of ALK each have established clinical value to select patients for targeted therapies (21). Undoubtedly similar success stories will emerge for new markers and novel therapies. Some global properties of the cancer genome, rather than specific combinations of genes, may also emerge as a new type of biomarker (22). For example, telomeric allelic imbalance is associated with greater sensitivity to DNA damaging agents (23). Our study demonstrates that prospective testing of multiple candidate biomarkers for the same drug can be performed in the context of a Phase II trial to build evidence for large scale validation in a definitive, larger randomize trial.

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Table 1. Patient characteristics and best response to therapy

	Cell Line Derived Predictor arm	SRC Pathway Activity arm	Dasatinib Target Index arm
Number of patients enrolled	9	8	13
Age (range)	46.5 years (33-69)	51.6 yeas (36-60)	54.3 years (42-62)
HER2-positive	0	1	0
ER- or PR-positive	1	7	2
Average number (and range) of metastatic sites	2(1-4)	2 (1-4)	2 (0-3)
Patients with > 3 lines of prior chemotherapy for metastatic disease	8	8	13
Patients with > 3 lines of prior endocrine therapy	1	7	1
Patients with prior anti-HER2 therapy	0	1	0
Number of patients positive for given marker*	9	9	19
Progression as best response	9	8	12
Clinical benefit	0	0	1 (stable disease)

* Individuals who were positive for more than one predictor were assigned to the marker arm for which they had the highest percentile positivity.

Figure Legends

Figure 1. Consort diagram of trial participants.

The figure shows the patient disposition for all 97 patients consented for the trial.

Figure 2. Kaplan Meier progression-free survival curves for the three marker arms.

Progression free survival is shown by marker arm for the treated patients measured in days from the date of starting therapy.

Figure 1: Consort diagram of trial participants

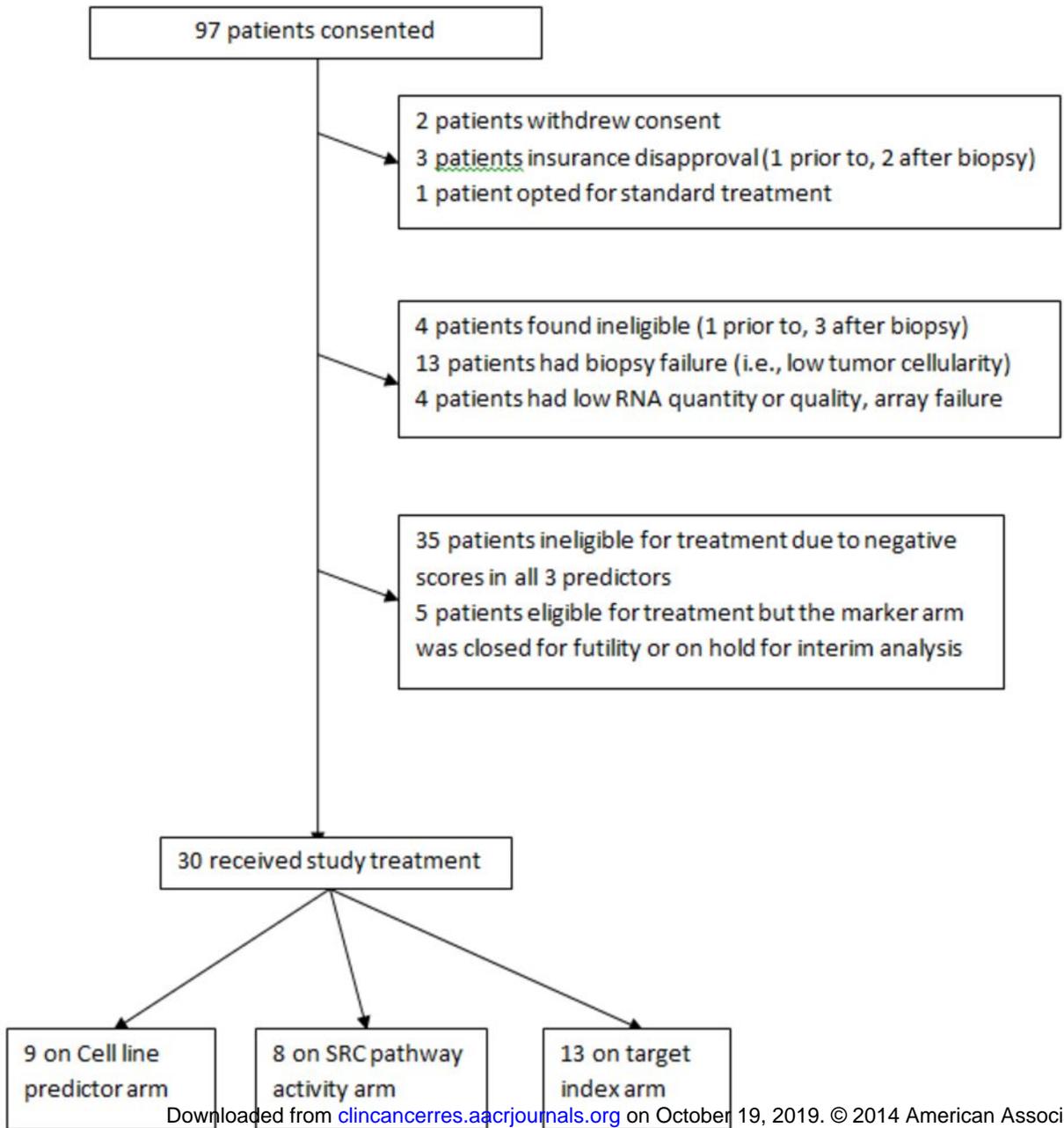
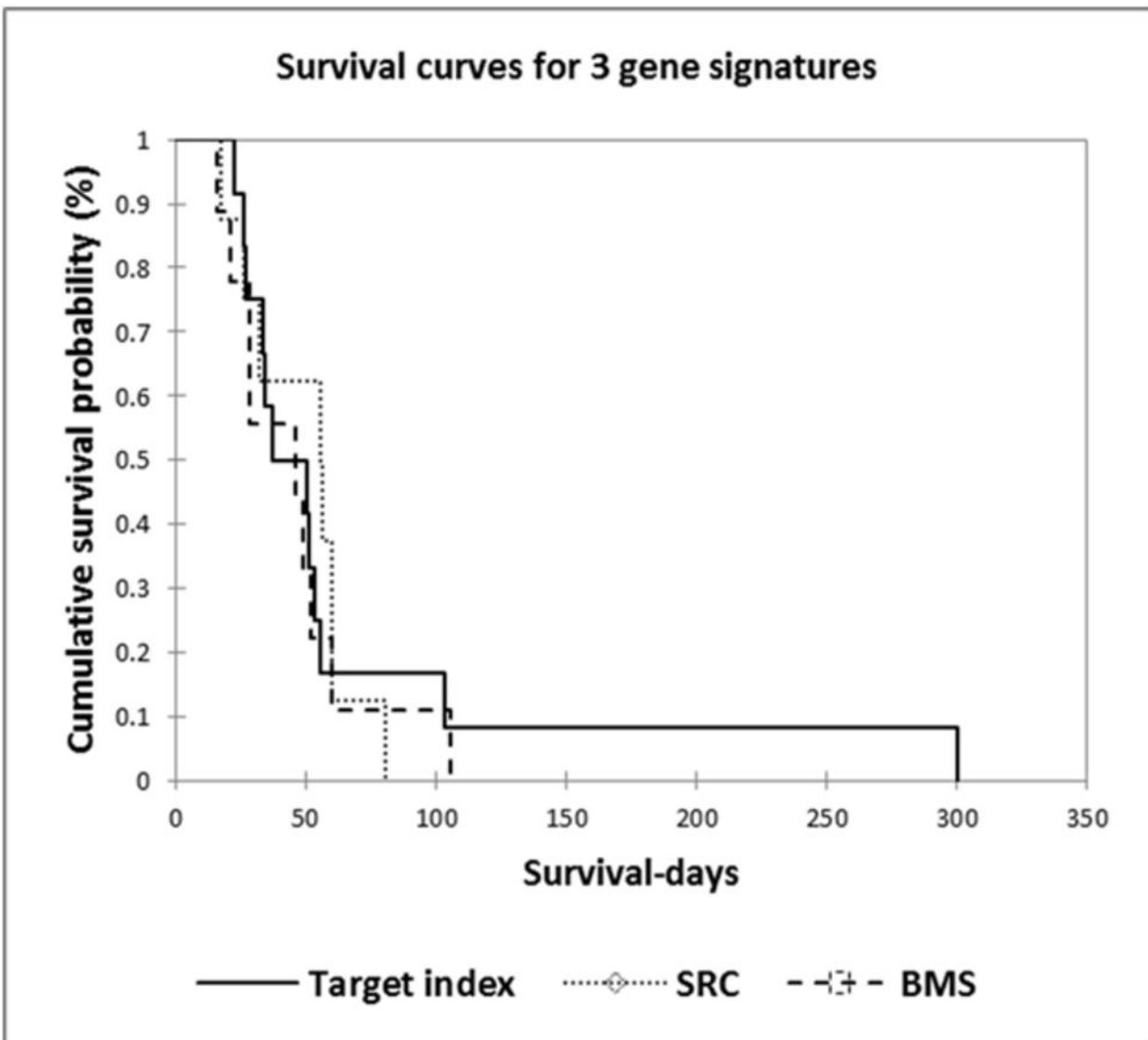


Figure 2: Kaplan Meier progression-free survival curves for the three marker arms.



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