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(54) Title: PREDICTORS FOR EVALUATING RESPONSE TO CANCER THERAPY

(57) Abstract: Embodiments of the invention include methods of evaluating a patient and determining the likelihood of a treatment outcome to one or four categories of residual cancer burden classification.



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DESCRIPTION

PREDICTORS FOR EVALUATING RESPONSE TO CANCER THERAPY

[0001] This application claims priority to U.S. Provisional Patent Application serial number 61/020,292 filed January 10, 2008, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

I. FIELD OF THE INVENTION

[0002] Embodiments of this invention are directed generally to biology and medicine. In certain aspects the invention relates to a gene set whose levels of expression are evaluated and used to prognose and/or derive a survival indicator for a patient who has undergone therapy, who is undergoing therapy, or who is a candidate for therapy.

II. BACKGROUND

[0003] There are four main approaches to improving the ability to predict responsiveness to therapies. One approach is a standard predictive or chemopredictive study focused on treatment, in which a sufficiently powered discovery population of subjects is used to define a predictive test that must then be proven to be accurate in a similarly sized validation population (Ransohoff, 2005; Ransohoff 2004). Several studies have used this approach to define predictive genes for adjuvant tamoxifen therapy (Ma *et al.*, 2004; Jansen *et al.*, 2005; Loi *et al.*, 2005). There are advantages to this approach, particularly when samples are available from mature studies for retrospective analysis. But two disadvantages are that the study design is empirical and that adjuvant (post surgery) treatment introduces surgery as a confounding variable, because it is impossible to ever know which patients were cured by their surgery and would never relapse, irrespective of their sensitivity to systemic therapy. Neoadjuvant chemotherapy trials enable a direct comparison of tumor characteristics with pathologic response to the specific therapy (Ayers *et al.*, 2004).

SUMMARY OF THE INVENTION

[0004] In medicine today, doctors search for methods of predicting how a patient (given their condition) may respond to treatment. Symptoms and tests may indicate favorable treatment with standard therapies. Likewise, a number of symptoms, health factors, and tests may indicate a less favorable treatment result with standard treatment - this may indicate that a more aggressive treatment plan may be desired. Prognostic scoring is also used for cancer outcome predictions. A "predictor score", "predictor index", or "index" or any variation of these terms, when used herein includes the computation of a numeric score obtained from the expression levels of the genes identified in Table 2, 4, or 6 multiplied by a prespecified positive or negative constant for each gene and added up to produce a single value. The value of the predictor is comparable to a reference score or scale form which assessment of the patient sample is made.

[0005] Although pathologic complete response (pCR) has been adopted as the primary endpoint for pre-operative (neoadjuvant) therapy trials because it is associated with long-term survival, it has not been uniformly or consistently defined (Bear, 2006; Carey, 2005; Hennessy, 2005; Kaufmann, 2006; Kuroi, 2005; Kurosumi, 2004; Rajan, 2004; von Minckwitz, 2005). While it is generally agreed that a definition of pCR should include patients without residual invasive carcinoma in the breast (pT0), the presence of nodal metastasis, minimal residual cellularity, and residual *in situ* carcinoma are not consistently stated as either pCR or residual disease (RD) (Bear, 2006; Kaufmann, 2006; Hennessy, 2005; Rajan, 2004). Therefore, dichotomization of response as pCR or residual disease (RD) may be simplistic for the objective of assay discovery and validation, particularly because residual disease (RD) after neoadjuvant treatment includes a broad range of actual tumor shrinkage. In some patients who are categorized as RD but actually show minimal residual disease, the response outcome blurs the prognostic distinction between pCR and RD. On the other hand, it should be possible to clearly identify patients within RD who are resistant to treatment in order to develop management strategies for this adverse outcome.

[0006] Expression markers are chosen for the ability to classify and/or identify patients as to probability for response (or non response) to therapy. Response to

therapy is commonly classified by the RECIST criteria established by the World Health Organization, the National Cancer Institute and the European Organization for Research and Treatment of Cancer. The RECIST criteria classify response as progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR). A good response is typically considered to include PR+CR (collectively referred to herein as Objective Response).

[0007] Certain aspects of the invention include methods of evaluating a cancer patient comprising one or more of the steps of (a) evaluating gene expression levels in a patient sample comprising cancer cells or an RNA sample isolated from one or more a patient samples, wherein a plurality of genes to be evaluated are selected from 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 125, 130, or all of the genes identified in Tables 2, 4, or 6 including all ranges and values there between and all subsets and combinations thereof (5, 10, 15, 20, 25, 100, 125, 130 or more such genes can be specifically excluded, including all values and ranges there between)(in certain aspects, the gene subset is selected based on the rank, e.g. in selecting a 10 gene subset genes ranked 1-10 are selected); (b) calculating a predictor score using a gene expression profile of selected markers; and (c) assessing the likelihood of a therapeutic outcome using the predictor score. The method may further comprise classifying a patient prior to evaluation. In certain aspects classification can include identifying a cancer patient with a disease state classified as a residual disease state or other clinically defined state prior to evaluation. In certain aspects, a predictor includes but is not limited to a measure for distant relapse-free survival (DRFS).

[0008] In still a further aspect, a gene expression profile or transcriptional profile comprises 5, 10, 20, 30, 60, 120, 125, 130, 135, 240, or all the genes of Table 2, 4 or 6 including all values and ranges there between as well as a number of subsets of these genes which may include some genes from one or more tables and exclude others from the same table or other tables.

[0009] In other aspects, a patient may be stratified or analyzed by using other factors such as protein expression, demographic information, family history, and other biological or medical states. The method may include determining Her2-neu and/or

estrogen receptor status of the patient sample and/or evaluation of tumor size, cellularity of tumor bed, and/or nodal burden to name a few.

[0010] The methods may also provide a treatment recommendation depending on the assessment derived from analysis of the gene expression profile as well as other factors. In certain aspects the recommendation may be based on residual cancer burden (RCB) classification or the like. A treatment is typically a standard treatment or a more aggressive non-standard treatment depending on the analysis. For example a treatment may be a combination of one or more cancer therapies, such as hormonal therapy and/or chemotherapy. Hormonal therapy includes, but is not limited to tamoxifen therapy, aromatase inhibitor therapy, or SERM therapy.

[0011] In other aspects, preparing a gene expression index or transcriptional profile can include one or more of the following steps: (a) obtaining data associated with a plurality of cancer patients, such as breast cancer, melanoma, ovarian cancer, testicular cancer or the like comprising measuring expression levels of a plurality of genes in samples from a plurality of patients; (b) partitioning the data into a first and second dataset; (c) evaluating the data and identifying data associated with a particular treatment outcome; (d) selecting a set of genes whose expression levels are indicative of therapeutic outcome. In one aspect, selecting a transcriptional profile includes evaluation of survival of the patient population sampled for all or part of the reference population of tumor samples, such as the distant relapse-free survival (DRFS) of the patient population.

[0012] Other aspects of the invention include kits to determine responsiveness of a cancer or cancer patient to a treatment or therapy comprising one or more of (a) reagents for determining expression levels of a plurality of genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof, such as probe sets that identify and measure the levels of gene transcripts, transcription, or protein levels; and (b) software encoding methods for designing, gathering, inputting, analyzing and/or assessing various data, which includes an algorithm for calculating a predictor score based on the analysis of the gene expression levels.

[0013] In still other aspects the invention includes an apparatus, or system for providing assessment of a sample relative to a gene expression profile, the system comprising (a) an application server comprising an input manager to receive expression data from a user for a plurality of genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof or subsets thereof obtained from a patient sample or an RNA sample from such patient sample; and (b) a network server comprising an output manager constructed and arranged to provide an assessment to the user.

[0014] In yet another aspect the invention includes a computer readable medium having software modules for performing the one or more of the methods described herein comprising the acts of: (a) comparing gene expression data obtained from a patient sample for a plurality of genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof with a reference; and (b) providing a predictor score to a physician for use in determining an appropriate therapeutic regimen for a patient.

[0015] In still yet another aspect the invention includes a computer system, having a processor, memory, external data storage, input/output mechanisms, a display, for performing the method of the invention, comprising (a) a database; (b) logic mechanisms in the computer for generating the transcriptional profile index; and (c) a comparing mechanism in the computer for comparing the gene expression reference to expression data from a patient sample or an RNA sample from such a patient sample to calculate a predictor score.

[0016] An internet accessible portal may be use to provide biological information constructed and arranged to execute a computer-implemented methods for providing: (a) a comparison of gene expression data of a plurality of genes of claim 1 in a patient sample with a transcriptional profile index; and (b) providing a predictor score to a physician for use in determining an appropriate therapeutic regime for a patient.

[0017] Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention

applies to other aspects of the invention as well and vice versa. The embodiments in the Example section are understood to be embodiments of the invention that are applicable to all aspects of the invention.

[0018] The terms “inhibiting,” “reducing,” or “prevention,” or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition to achieve a desired result.

[0019] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0020] Throughout this application, the term “about” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0021] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[0022] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0023] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

[0024] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0025] **FIG. 1** Characteristics of the patient cohort used in developing the predictors shown with stratification by ER status and Her-2 status.

[0026] **FIG. 2.** Schematic of predictor development and evaluation protocol employing an external cross-validation loop to validate the predictor performance while accounting for selection bias and an internal cross-validation loop to optimize gene selection and parameter tuning. A Monte-Carlo step repeats the external cross-validation process several times to obtain average performance.

[0027] **FIG. 3** Predictive performance of RCB-0 predictor on Her2-normal and Her2-amplified patients.

[0028] **FIG. 4** Predictive performance of RCB-0/I predictor on Her2-normal and Her2-amplified patients.

[0029] **FIG. 5** Plot of survival outcomes in predicted responders by the RCB-0/I predictor

[0030] **FIG. 6** Plot of actual patient outcomes measured as distant relapse-free survival in responders predicted by RCB-0/I predictor stratified by ER status.

[0031] **FIG. 7** Predictive performance of RCB-0/I predictor developed for sample robustness in Her2-normal patients.

[0032] **FIG. 8** Plot of actual patient outcomes measured as distant relapse-free survival in responders predicted by RCB-0/I gene predictor.

[0033] **FIG. 9** Plot of actual patient outcomes measured as distant relapse-free survival in responders predicted by RCB-0/I predictor stratified by ER status.

DETAILED DESCRIPTION OF THE INVENTION

[0034] Despite the critical importance of selecting the most effective adjuvant/neoadjuvant chemotherapy for an individual, diagnostic tests to guide selection of the optimal regimen for a particular patient continue to be inadequate (Carlson, 2000; Goldhirsch, 2003); Estrogen receptor (ER) negative status, high grade and high proliferative activity are histological characteristics that tend to indicate a more chemotherapy sensitive cancer (Bast, 2001; Ross, 2003; Rouzier, 2005). However, although these clinicopathologic variables may identify eligibility or predict general chemotherapy sensitivity, they have little potential to guide selection of a specific treatment regimen in standard-of-care practice.

[0035] The limited utility of individual markers to predict clinical outcome of cancer may be due to the incomplete understanding of the function of these markers. In addition, biologically important molecules act in concert and form complex, interactive pathways where an individual molecule may only contribute limited information on the functional activity of a whole pathway. The promise of microarray technology is that, by assessing the transcriptional activity of a large number of genes, the complex gene-expression profile may contain more information than any individual marker that contributes to it.

[0036] There are examples indicating that the molecular classification of cancer based on gene-expression profiles could be important in framing patient management strategies. Unsupervised clustering of breast cancer specimens consistently separated tumors into ER positive and ER negative clusters (Gruvberger, 2001; Perou, 2000; Pusztai, 2003). Analysis of gene-expression profiles also distinguished sporadic breast cancers from breast cancer gene, *BRCA*, mutant cases (Hedenfalk, 2001). Transcriptional profiles have also revealed previously unrecognized molecular subgroups within existing histological categories in breast cancer (Perou, 2000), diffuse large-B-cell lymphoma, and soft tissue and central nervous system embryonal tumors (Nielsen, 2002; Pomeroy, 2002). In addition, gene-expression profiles have been shown to predict survival of patients with node-negative breast cancer (van de Vijver, 2002; van 't Veer, 2002), lymphoma (Alizadeh, 2000; Rosenwald, 2002), renal cancer (Takahashi, 2001), and lung cancer (Beer, 2002).

[0037] Previous efforts into applying gene expression-based predictors in breast cancer have focused largely on predicting a patient's risk of cancer recurrence in the event of either receiving no systemic treatment after surgery (van de Vijver, 2002; van 't Veer, 2002; Wang, 2005) or receiving tamoxifen, a hormonal therapy agent, for 5 years after surgery (Paik, 2006; Paik, 2004; Ma, 2006; Davis, 2007). These gene-based predictors do not directly address the need or the responsiveness to chemotherapy although a high risk of recurrence may indirectly suggest the general consideration of chemotherapy among the available options for patient management.

[0038] Other research efforts have also reported gene-based predictors of response to standard breast cancer treatments (Ayers, 2004; Bild, 2006; Chang, 2003; Hess, 2006; Modlich, 2006) although these are not commercially marketed yet as assays. Some of these predictors are developed using patient tissue samples treated clinically with a specific chemotherapy regimen and subsequently comparing genomic profiles of responders versus non-responders using survival-driven endpoints (Ayers, 2004; Chang, 2003; Hess, 2006; Modlich, 2006) whereas others are focused on analyses of changes in genes within breast cancer cell lines that are treated *in vitro* with single standard therapeutic agents (Bild, 2006).

[0039] As an *in vivo* model for marker development and validation, pre-operative (neoadjuvant) chemotherapy provides an opportunity to gain access to samples that directly describe tumor response to therapy. Furthermore, complete eradication of all invasive cancer from the breast and regional lymph nodes, called pathologic complete response (pCR), is associated with excellent long-term cancer-free survival (Fisher, 1998; Kuerer, 1999). Therefore, the goal in developing treatment-directed response markers is to evaluate gene expression profiles in order to predict who may achieve pCR versus residual disease (RD). Pathologic CR is a meaningful clinical end-point to predict because these patients experience prolonged disease-free and overall survival compared to patients with lesser response (Cleator, 2005; Fisher, 1998; Kaufmann, 2006; Wolmark, 2001). Good survival in these patients reflects benefit from chemotherapy since most clinical and gene expression variables that are associated with pCR (*i.e.*, high grade, ER-negative status, high Oncotype DX recurrence score) tend to predict worse prognosis in the absence of chemotherapy (Paik, 2006; Paik, 2004).

[0040] Previous work has demonstrated the development of a 30-probe genomic predictor for response to a taxane-containing chemotherapy (Ayers, 2004; Hess, 2006). The treatment administered in the neoadjuvant setting was sequential paclitaxel anthracycline preoperative chemotherapy (T/FAC). A multidrug regimen was selected for study because combination chemotherapy represents the current clinical standard for patients who require systemic cytotoxic treatment. Also, studies that explore gene signatures for response to individual drugs may not fully capture sensitivity to combination chemotherapy as practiced in standard-of-care.

[0041] A cohort of 82 patients was used for predictor discovery of pCR to preoperative T/FAC chemotherapy using fine needle biopsies taken before treatment and by analyzing gene profiles generated from a commercially available standard gene expression profiling technology (Affymetrix, Santa Clara, CA). Although several analytic techniques and resulting gene sets for response prediction were studied, the nominally best predictor for pCR with the least number of genes, called DLDA-30, was selected for independent validation in 51 additional patients. The predictor showed substantially higher sensitivity (a measure of how well a predictor identifies responsiveness or non-responsiveness to a therapy, *e.g.*, true positives / (true positives + false negatives)) (92% vs 61%) and slightly better negative predictive value (NPV, the proportion of patients with negative test results who are correctly diagnosed.) (96% vs 86%) than a clinical predictor based on ER, grade and age (Hess, 2006). The positive predictive value (PPV, is the proportion of patients with positive test results who are correctly diagnosed.) of the genomic predictor at 52% (95 CI: 30%-73%), was significantly higher than the baseline 26% pCR rate in unselected patients. A sensitivity of 100% means that the test recognizes all patient as either responsive to therapy or non-responsive to therapy. Typically, sensitivity alone does not tell us how well the test predicts other classes (that is, about the negative cases). Sensitivity is not the same as the positive predictive value (ratio of true positives to combined true and false positives), which is as much a statement about the proportion of actual positives in the population being tested as it is about the test. The calculation of sensitivity typically does not take into account indeterminate test results. If a test cannot be repeated, the options are to exclude indeterminate samples from analyses (but the number of exclusions should be stated when quoting

sensitivity), or, alternatively, indeterminate samples can be treated as false negatives (which gives the worst-case value for sensitivity and may therefore underestimate it).

[0042] Although this predictor and others described in literature (Chang, 2003; Modlich, 2006) may help define a patient population that is more likely to achieve pCR than the general patient population, further developments can help refine prediction of treatment response considerably. Although pCR as a response endpoint is strongly correlated with high treatment-related survival, patients with residual disease (RD) after treatment encompass a wide range of outcomes ranging from very good prognosis (“near-pCR”) to drug resistance. Furthermore, refinements can also be directed at incorporating tumor heterogeneity by accounting for marker status such as ER and Her2 within predictor development. Predictors that can better classify response outcomes to capture and differentiate the high responders and non-responders within the spectrum of residual disease, and capture the heterogeneity of tumors, could significantly benefit patient management.

[0043] Although pathologic complete response (pCR) has been adopted as the primary endpoint for neoadjuvant trials because it is associated with long-term survival, it has not been uniformly or consistently defined (Bear, 2006; Carey, 2005; Hennessy, 2005; Kaufmann, 2006; Kuroi, 2005; Kurosumi, 2004; Rajan, 2004; von Minckwitz, 2005). While it is generally agreed that a definition of pCR should include patients without residual invasive carcinoma in the breast (pT0), the presence of nodal metastasis, minimal residual cellularity, and residual *in situ* carcinoma are not consistently stated as either pCR or residual disease (RD) (Bear, 2006; Kaufmann, 2006; Hennessy, 2005; Rajan, 2004). Therefore, dichotomization of response as pCR or residual disease (RD) may be simplistic for the objective of assay discovery and validation, particularly because residual disease (RD) after neoadjuvant treatment includes a broad range of actual tumor shrinkage. In some patients who are categorized as RD but actually show minimal residual disease, the response outcome blurs the prognostic distinction between pCR and RD. On the other hand, it should be possible to clearly identify patients within RD who are resistant to treatment in order to develop management strategies for this adverse outcome.

[0044] A measure of residual disease or residual cancer burden (RCB), previously developed and reported, may be useful as a variable to characterize response to

treatment (Symmans, 2007). This measure is derived from the primary tumor dimensions, cellularity of the tumor bed, and axillary nodal burden. Each component contributes meaningful pathologic information and can be obtained using routine pathologic materials and methods of interpretation that could easily be implemented in routine diagnostic practice. RCB measurements can provide a continuous parameter of residual disease and thus of response, so that all subject responses contribute to the analysis.

[0045] RCB is divided into four survival-related classes (RCB-0 to RCB-III) where patients with minimal residual disease (RCB-I) have the same 5-year relapse-free survival as those with pCR (RCB-0), irrespective of the type of neoadjuvant chemotherapy administered, adjuvant hormonal therapy or the pathologic stage of RD. Therefore, the combination of RCB-0 (pCR) and RCB-I expands the subset of patients who can be identified as having “good response” and to have benefited from the chemotherapy. Extensive residual disease (RCB-III), on the other hand, is associated with poor prognosis, irrespective of the type of neoadjuvant chemotherapy administered, adjuvant hormonal therapy, or the pathologic stage of RD. In particular, all patients with RCB-III after T/FAC chemotherapy, who did not receive adjuvant hormonal therapy, suffered distant relapse within 3 years (Symmans, 2007). This identifies an important subset of patients who are not responsive to chemotherapy, or with residual disease (after surgery) that is too extensive to be controlled by hormonal therapy alone.

[0046] Therefore, residual cancer burden (RCB) is an informative tool and a metric to help develop response predictors based on better characterization of likely treatment outcomes. RCB categories can be employed with existing methods to define surrogate endpoints from neoadjuvant trials. As a metric correlated with survival, RCB is strongly and independently prognostic and the classes of RCB capture distinct sets of survival-based outcomes. Development of a predictor that reports likelihood of a patient’s tumor post-treatment to belong to one of the RCB classes, rather than simply pCR as an endpoint, can yield valuable diagnostic information for efficient treatment management. In certain aspects, predictors specific to RCB-0 (pCR or complete response), RCB-0/I (pCR+near-pCR called good response) and RCB-III (resistance) are developed. In certain aspects of the methods

described, the inventors have also accounted for tumor sub-types based on the status of two receptors, Her2-neu and ER, allowing for the predictors to capture heterogeneity within breast cancers and achieve acceptable diagnostic performance.

I. PREDICTORS OF RESPONSE TO THERAPY

[0047] Sets of genes are defined that are prognostic, diagnostic, or predictive or indicative of the outcome for a cancer patient. These genes can be incorporated into an index or predictor of such an outcome and used in the management of the treatment for a given patient. Prognosis is a medical term denoting the doctor's prediction of how a patient's disease will progress, and whether there is chance of recovery.

[0048] Outcome can be represented in various forms to indicate probability of survival or the likely survival outcome after surgery and therapy. In biostatistics, survival rate is a part of survival analysis, indicating the percentage of people in a study or treatment group who are alive for a given period of time after diagnosis. Survival rates are important for prognosis; for example, whether a type of cancer has a good or bad prognosis can be determined from its survival rate or survival outcome.

[0049] Patients with a certain disease can die directly from that disease or from an unrelated cause such as a car accident. When the precise cause of death is not specified, this is called the overall survival rate or observed survival rate. Doctors often use mean overall survival rates to estimate the patient's prognosis. This is often expressed over standard time periods, like one, five, and ten years. For example, prostate cancer has a much higher one year overall survival rate than pancreatic cancer, and thus has a better prognosis.

[0050] When someone is more interested in how survival is affected by the disease, there is also the net survival rate, which filters out the effect of mortality from other causes than the disease. Typically, the two main ways to calculate net survival are relative survival and cause specific survival or disease specific survival.

[0051] Relative survival is calculated by dividing the overall survival after diagnosis of a disease by the survival as observed in a similar population that was not diagnosed with that disease. A similar population is composed of individuals with at

least age and gender similar to those diagnosed with the disease. Cause specific survival is calculated by treating deaths from other causes than the disease as withdrawals from the population that don't lower survival, comparable to patients who are not observed any longer, e.g. due to reaching the end of the study period. Relative survival has the advantage that it does not depend on accuracy of the reported cause of death; cause-specific survival has the advantage that it does not depend on the ability to find a similar population of people without the disease.

[0052] Survival is not the only endpoint that can be used as a metric in developing predictors such as those described herein. Endpoints or therapeutic outcomes can include survival or distant relapse-free survival (DRFS). Other endpoints are discussed in Cooper and Kaanders, Biological surrogate end-points in cancer trials: Potential uses, benefits and pitfalls, European Journal of Cancer, Volume 41, Issue 9, Pages 1261-1266, which is incorporated herein by reference. A "surrogate marker" or "surrogate endpoint" or "secondary endpoint" typically will refer to a biological or clinical parameter that is measured in place of the biologically definitive or clinically most meaningful parameter, *i.e.*, survival. Primary endpoints may also include limitation of pharmacologic therapies, reduction of time to death, or reduction in the progression of the disease, disorder, or condition. Surrogate markers are pathophysiologic parameters determined by medical or clinical laboratory diagnosis that are associated and have been correlated with the prognosis, progression, predisposition, or risk analysis with a disease, disorder, or condition that are not directly related to the primary diagnosed pathophysiologic condition. Secondary endpoints are those that supplement the primary endpoint. For example, secondary endpoints include reduction in pharmacologic therapy, reduction in requirement of a medical device, or alteration of the progression of the disease disorder, or condition. Typically, a clinical endpoint may refer to a disease, symptom, or sign that constitutes one of the target outcomes of the therapy or clinical trial. The results of a therapy or clinical trial generally indicate the number of people enrolled who reached the pre-determined clinical endpoint during the study interval, compared with the overall number of people who were enrolled. Once a patient reaches the endpoint, he or she is generally excluded from further experimental intervention (the origin of the term endpoint). For example, a clinical trial investigating the ability of a medication to prevent heart attack might use chest pain as a clinical endpoint. Any patient enrolled

in the trial who develops chest pain over the course of the trial, then, would be counted as having reached that clinical endpoint. The results would ultimately reflect the fraction of patients who reached the endpoint of having developed chest pain, compared with the overall number of people enrolled. When an experiment involves a control group, the fraction of individuals who reach the clinical endpoint after an intervention is compared with the fraction of individuals in the control group who reached the same clinical endpoint, thus reflecting the ability of the intervention to prevent the endpoint in question. Some studies will examine the incidence of a combined endpoint, which can merge a variety of outcomes into one group.

[0053] When building prediction rules of treatment response or disease state in general from gene expression data can be selected from a small subset of informative genes that will be used as prognostic features in the predictor. Many predictors employ univariate filtering to rank the candidate genes according to the p-value of a two-sample unequal variance t-test comparing the mean expression values of each gene in the two response classes (*e.g.*, pCR and RD). Univariate filtering methods have the disadvantage that they do not deal well with redundant features (genes that have similar expression profiles) and therefore the resulting predictors tend to be less robust (Lai, 2006).

[0054] The method used to identify predictive genes involved first, applying one or more filters to the gene expression data of all probes on an array to select the top probe sets to be used in signature development using the above described algorithm. Gene filtering can be based on the regularized t-test for the selected response endpoint such as RCB-0 (same as pathologic complete response), or RCB-0/I (very good response). Other methods for gene filtering include methods that utilize non-specific global filtering criteria. These include, but are not limited to *intensity-based filtering*, which aims to remove genes that are not expressed at all in the samples studied or *variability-based filtering*, which aims to remove genes with low variability across samples.

[0055] A multivariate method was used to simultaneously select the signature genes and to calculate the predictor score. The final predictor is determined by level of penalization, which determines the number of genes included in the predictive signature, and the choice of a decision threshold to dichotomize the classification

score into a good prognosis and a bad prognosis group. As one example, the inventors selected the maximum level of penalization resulting in the smallest signatures that yield significant cross-validated predictor or outcome predictor, each of these terms can be used interchangeably, performance – this step determines the signature probe sets and their weights. Then, a decision threshold is selected in order to optimize the predictive values of the predictor. Evaluation of the predictors was based on the joint confidence interval of the positive predictive value (PPV) and the negative predictive value (NPV) of the predictor at 5% significance level (low 95% confidence limit of $PPV \geq \text{baseline response rate}$ and low 95% confidence limit of $NPV \geq 1 - \text{baseline response rate}$).

[0056] In developing the RCB-based predictor, the inventors used an approach that combines feature selection and model discovery using a multivariate penalized approach, an example of which is Gradient Directed Regularization developed by Prof. J. Friedman at Stanford University, a description of which can be found on the World Wide Web at stat.stanford.edu/~jhf/ftp/pathlite.pdf. The informative genes are selected with penalization using the maximization of the area under the receiver operating characteristic (ROC) curve (AUC) as the optimization criterion. Ma and Huang have previously used a similar approach for disease classification (Ma, 2006). A receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot of the sensitivity vs. (1 - specificity) for a binary classifier system as its discrimination threshold is varied. The ROC can also be represented equivalently by plotting the fraction of true positives (TPR = true positive rate) vs. the fraction of false positives (FPR = false positive rate). The best possible prediction method would yield a point in the upper left corner or coordinate (0,1) of the ROC space, representing 100% sensitivity (all true positives are found) and 100% specificity (no false positives are found). The (0,1) point is also called a perfect classification. A completely random guess would give a point along a diagonal line (the so-called line of no-discrimination) from the left bottom to the top right corners. The diagonal line divides the ROC space in areas of good or bad classification/diagnostic. Points above the diagonal line indicate good classification results, while points below the line indicate wrong results.

[0057] As an example of predictor discovery and evaluation the protocol suggested by Wessels *et al.* was followed (Wessels, 2005). The methodology is shown in FIG. 2 and briefly explained below. First, the input dataset is randomly partitioned into a training set and a test set. A 3-fold cross-validation based on Dudoit *et al.* recommendation of a 2:1 split between training and test sets was used (Dudoit, 2002). The training set consisting of 2/3 of the original data is used to develop a predictor. To account for bias in the several data-dependent decisions involved in building the predictor, a 5-fold internal cross-validation can be used to select the optimal set of genes for the predictor and to tune the parameters of the predictor, *e.g.*, the degree of penalization. Since different optimal reporter gene sets might result from the different internal cross-validation folds, the number of times each gene is selected is tracked to provide a measure of its importance or its reliability. The trained predictor is then tested on the 1/5 hold-out part of the training dataset and its performance is evaluated based on the AUC.

[0058] To obtain a less biased estimate of classification performance, the trained predictor or outcome predictor can be evaluated on the test set (1/3 of the original data) that was not used in training the predictor. To assess the significance of the predictive performance of the trained predictor, the permutation predictive performance of the predictor was estimated by randomly scrambling the outcome labels in the test dataset. The entire process of randomly splitting the data to a training and a test set was repeated a number of times to obtain the distributions and summary statistics of the performance metrics.

[0059] Typically, under cross-validation the decision threshold is varied along all possible values and for each value predictor performance (accuracy, positive predictive value (PPV), negative predictive value (NPV)) is determined. The threshold is selected that yields the best compromise between PPV and NPV, as typically increasing PPV results in decreasing NPV. Typically, the objective is to maximize both.

[0060] In certain aspects, other measurements or determinations can be made in conjunction with the nucleic acid analysis, for example determination of protein expression and/or histology of a sample. Protein expression can be detected in tumor tissue, cell material obtained by biopsy and the like. For example, a biopsy sample

can be immobilized and contacted with an antibody, an antibody fragment or an aptamer that binds selectively to the protein to be detected. The sample can be assayed to determine whether the antibody, fragment or aptamer has bound to the protein by techniques well known in the art. Protein expression can be measured by a variety of methods including but not limited to Western blot, immunoblot, enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), immunoprecipitation, surface plasmon resonance, immunohistochemical (IHC) analysis, mass spectrometry, fluorescence activated cell sorting (FACS) and flow cytometry.

[0061] In a further aspect, IHC analysis is used to measure protein expression. The level of expression for a sample is determined by IHC by staining the sample for a particular expression marker and developing a score for the staining. For example, monoclonal antibodies can be used to stain for the expression of a marker of interest. Mouse antibodies are known for use in the staining of the marker PTEN. Samples can be evaluated for the frequency of cells stained for each sample and the intensity of the stain. Typically, a score based on the frequency (rated from 0-4) and intensity (rated from 0-4) of the stained sample is developed as a measure of overall expression. Exemplary but non-limiting methods for IHC and criteria for scoring expression are described in detail in Handbook of Immunohistochemistry and In Situ Hybridization in Human Carcinomas, M. Hayat Ed., 2004, Academic Press.

II. USE OF PREDICTOR FOR PATIENT EVALUATION

[0062] In one aspect of the invention, a predictor or transcriptional profile index is used to measure the expression of many genes that provide predictive information about a likely outcome for a particular patient. The invention includes the methods for standardizing the expression values of future samples to a normalization standard that will allow direct comparison of the results to past samples, such as from a clinical trial. The invention also includes the biostatistical methods to calculate and report such results. A sample as used herein can comprise any number of cells that is sufficient for a clinical diagnosis or prognosis, and typically contain at least, at most or about 100 target cells.

[0063] Microarrays provide a suitable method to measure gene expression from clinical samples. mRNA levels measured by microarrays, such as Affymetrix U133A

gene chips, in fine needle aspirates (FNA), core needle biopsy, resected tissue samples from excised tumor and/or frozen tumor tissue samples of breast cancer correlated closely with protein expression by enzyme immunoassay and by routine immunohistochemistry.

[0064] *Estrogen receptor and Her2-neu status.* ER-positive breast cancer includes a continuum of ER expression that might reflect a continuum of biologic behavior and endocrine sensitivity. Others have reported that some breast cancers are difficult to predict as ER-positive based on transcriptional profile and described non-estrogenic growth effects, such as HER-2, more frequently in this small subset of tumors with aggressive natural history (Kun *et al.*, 2003). Indeed, ER mRNA levels are lower in breast cancers that are positive for both ER and HER2 (Konecny *et al.*, 2003).

III. CANCER THERAPIES

[0065] Diagnostic tools are needed not merely for prognosis, but, for providing a biological rationale and to demonstrate clinical benefit when they are used to guide the selection and duration of therapies, particularly in light of the cost, complexity, toxicity, benefits and other factors related to such therapies. An index or predictor can be used to predict the likelihood of response rather than intrinsic prognosis.

[0066] In addition to other known methods of cancer therapy, hormone therapies may be employed in the treatment of patients identified as having hormone sensitive cancers. Hormones, or other compounds that stimulate or inhibit these pathways, can bind to hormone receptors, blocking a cancer's ability to get the hormones it needs for growth. By altering the hormone supply, hormone therapy can inhibit growth of a tumor or shrink the tumor. Typically, these cancer treatments only work for hormone-sensitive cancers. If a cancer is hormone sensitive, a patient might benefit from hormone therapy as part of cancer treatment. Sensitive to hormones is usually determined by taking a sample of a tumor (biopsy) and conducting analysis in a laboratory.

A. Chemotherapy

[0067] Chemotherapy is the use of chemical substances to treat disease. In its modern-day use, it refers to cytotoxic drugs used to treat cancer or the combination of these drugs into a standardized treatment regimen. There are a number of strategies in

the administration of chemotherapeutic drugs used today. Chemotherapy may be given with a curative intent or it may aim to prolong life or to palliate symptoms.

[0068] Combined modality chemotherapy is the use of drugs with other cancer treatments, such as radiation therapy or surgery. Combination chemotherapy is a similar practice which involves treating a patient with a number of different drugs simultaneously, *e.g.*, T/FAC therapy. Typically, the drugs differ in their mechanism and side effects. The biggest advantage is minimizing the chances of resistance developing to any one agent.

[0069] In neoadjuvant chemotherapy (preoperative treatment) initial chemotherapy is aimed for shrinking the primary tumor, thereby rendering local therapy (surgery or radiotherapy) less destructive or more effective.

[0070] Adjuvant chemotherapy (postoperative treatment) can be used when there is little evidence of cancer present, but there is risk of recurrence. This can help reduce chances of resistance developing if the tumor does develop. It is also useful in killing any cancerous cells which have spread to other parts of the body. This is often effective as the newly growing tumors are fast-dividing, and therefore very susceptible.

[0071] Palliative chemotherapy is given without curative intent, but simply to decrease tumor load and increase life expectancy. For these regimens, a better toxicity profile is generally expected.

[0072] All chemotherapy regimens require that the patient be capable of undergoing the treatment. Performance status is often used as a measure to determine whether a patient can receive chemotherapy, or whether dose reduction is required.

[0073] In certain embodiments the chemotherapy is a taxane chemotherapy. The taxanes are diterpenes produced by the plants of the genus *Taxus* (yews). As their name suggests, they were first derived from natural sources, but some have been synthesized artificially. Taxanes include, but is not limited to Docetaxel, Larotaxel, Ortaxel, Paclitaxel, and Tasetaxel. Taxanes prevent growth of cancer cells by inhibiting the breakdown of microtubules, which normally occurs once a cell stops

dividing. Thus, treated cells become so clogged with microtubules that they cannot grow and divide.

[0074] Paclitaxel (Taxol) is isolated from the bark of the ash tree, *Taxus brevifolia*. It binds to tubulin (at a site distinct from that used by the vinca alkaloids) and promotes the assembly of microtubules. It has activity against malignant melanoma and carcinoma of the ovary. Maximal doses are 30 mg/m² per day for 5 days or 210 to 250 mg/m² given once every 3 weeks. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention. Docetaxel, a compound that is similar to paclitaxel, and is also used to treat cancer. Docetaxel comes from the needles of the yew tree. The FDA has approved docetaxel to treat advanced breast, lung, and ovarian cancer.

B. Hormone therapy

[0075] Several malignancies respond to hormonal therapy. Strictly speaking, this is not chemotherapy. Cancer arising from certain tissues, including the mammary and prostate glands, may be inhibited or stimulated by appropriate changes in hormone balance. Cancers that are most likely to be hormone-receptive include: Breast cancer, Prostate cancer, Ovarian cancer, and Endometrial cancer. Not every cancer of these types is hormone-sensitive, however. That is why the cancer must be analyzed to determine if hormone therapy is appropriate.

[0076] Steroids (often dexamethasone) can inhibit tumor growth or the associated edema (tissue swelling), and may cause regression of lymph node malignancies.

[0077] Prostate cancer is often sensitive to finasteride, an agent that blocks the peripheral conversion of testosterone to dihydrotestosterone.

[0078] Breast cancer cells often highly express the estrogen and/or progesterone receptor. Inhibiting the production (with aromatase inhibitors) or action (with tamoxifen) of these hormones can often be used as an adjunct to therapy.

[0079] Gonadotropin-releasing hormone agonists (GnRH), such as goserelin possess a paradoxical negative feedback effect followed by inhibition of the release of FSH (follicle-stimulating hormone) and LH (luteinizing hormone), when given

continuously. Some other tumors are also hormone dependent, although the specific mechanism is still unclear.

[0080] Hormone therapy may be used in combination with other types of cancer treatments, including surgery, radiation and chemotherapy. A hormone therapy can be used before a primary cancer treatment, such as before surgery to remove a tumor. This is called neoadjuvant therapy. Hormone therapy can sometimes shrink a tumor to a more manageable size so that it's easier to remove during surgery.

[0081] Hormone therapy is sometimes given in addition to the primary treatment — usually after — in an effort to prevent the cancer from recurring (adjuvant therapy). In some cases of advanced (metastatic) cancers, such as in advanced prostate cancer and advanced breast cancer, hormone therapy is sometimes used as a primary treatment.

[0082] Hormone therapy can be given in several forms, including: (A) Surgery -- Surgery can reduce the levels of hormones in your body by removing the parts of your body that produce the hormones, including: Testicles (orchiectomy or castration), Ovaries (oophorectomy) in premenopausal women, Adrenal gland (adrenalectomy) in postmenopausal women, Pituitary gland (hypophysectomy) in women. Because certain drugs can duplicate the hormone-suppressive effects of surgery in many situations, drugs are used more often than surgery for hormone therapy. And because removal of the testicles or ovaries will limit an individual's options when it comes to having children, younger people are more likely to choose drugs over surgery. (B) Radiation -- Radiation is used to suppress the production of hormones. Just as is true of surgery, it's used most commonly to stop hormone production in the testicles, ovaries, and adrenal and pituitary glands. (C) Pharmaceuticals -- Various drugs can alter the production of estrogen and testosterone. These can be taken in pill form or by means of injection.

[0083] The most common types of drugs for hormone-receptive cancers include: (1) Anti-hormones that block the cancer cell's ability to interact with the hormones that stimulate or support cancer growth. Though these drugs do not reduce the production of hormones, anti-hormones block the ability to use these hormones. Anti-hormones include the anti-estrogens tamoxifen (Nolvadex) and toremifene (Fareston) for breast

cancer, and the anti-androgens flutamide (Eulexin) and bicalutamide (Casodex) for prostate cancer. (2) Aromatase inhibitors -- Aromatase inhibitors (AIs) target enzymes that produce estrogen in postmenopausal women, thus reducing the amount of estrogen available to fuel tumors. AIs are only used in postmenopausal women because the drugs can't prevent the production of estrogen in women who haven't yet been through menopause. Approved AIs include letrozole (Femara), anastrozole (Arimidex) and exemestane (Aromasin). (3) Luteinizing hormone-releasing hormone (LH-RH) agonists and antagonists -- LH-RH agonists — sometimes called analogs — and LH-RH antagonists reduce the level of hormones by altering the mechanisms in the brain that tell the body to produce hormones. LH-RH agonists are essentially a chemical alternative to surgery for removal of the ovaries for women, or of the testicles for men. Depending on the cancer type, one might choose this route if they hope to have children in the future and want to avoid surgical castration. In most cases the effects of these drugs are reversible. Examples of LH-RH agonists include: Leuprolide (Lupron, Viadur, Eligard) for prostate cancer, Goserelin (Zoladex) for breast and prostate cancers, Triptorelin (Trelstar) for ovarian and prostate cancers and abarelix (Plenaxis).

[0084] One class of pharmaceuticals is the Selective Estrogen Receptor Modulators or SERMs. SERMs block the action of estrogen in the breast and certain other tissues by occupying estrogen receptors inside cells. SERMs include, but are not limited to tamoxifen (the brand name is Nolvadex, generic tamoxifen citrate); Raloxifene (brand name: Evista), and toremifene (brand name: Fareston).

IV. KITS

[0085] Further embodiments of the invention include kits for the measurement, analysis, and reporting of gene expression and transcriptional output. A kit may include, but is not limited to microarray, quantitative RT-PCR, antibodies, labeling or other reagents and materials, as well as hardware and/or software for performing at least a portion of the methods described. For example, custom microarrays or analysis methods for existing microarrays are contemplated. Also, methods of the invention include methods of accessing and using a reporting system that compares a single result to a scale of clinical trial results. In yet still further aspects of the invention, a digital standard for data normalization is contemplated so that the assay

result values from future samples would be able to be directly compared with the assay value results from past samples, such as from specific clinical trials.

V. EXAMPLES

[0086] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

EXAMPLE 1

Material and Methods

[0087] *Patients and samples* - Patient samples were collected at the Nellie B. Connally Breast Center of the University of Texas M. D. Anderson Cancer Center (MDACC) in Houston, Texas and at the Instituto Nacional de Enfermedades Neoplásicas (INEN) in Lima, Peru. During this research, patients were consented to undergo pretreatment fine needle aspiration (FNA) of the primary breast tumor or ipsilateral axillary metastasis before starting chemotherapy as part of an ongoing pharmacogenomic marker discovery program (Ayers, 2004; Hess, 2006). The aspiration was performed using a 23 or 25-gauge needle. Cells from 2-3 passes were collected into vials containing 1 ml of RNeasy lysis solution (Qiagen, Crawley, UK) and stored at -80°C . FNA samples on average contain 80% neoplastic cells and contain little or no stromal cells or normal breast epithelium. Gene expression data generated from FNAs captures the molecular characteristics of the invasive cancer including the molecular class (Pusztai, 2003). At least 70% of all aspirations yielded at least 1 μg total RNA that is required for the gene expression profiling. The main reason for failure to obtain sufficient RNA was acellular aspirations. Two hundred and twenty seven (227) consecutively accrued patients with at least 1 μg RNA were included in

this analysis. All patients received 24 weeks of sequential paclitaxel and FAC preoperative chemotherapy (T/FAC). Metallic markers were placed under radiological guidance in the shrinking tumor bed for any patient interested in breast conserving surgery whose tumor became < 2 cm by imaging during the course of treatment. At the completion of neoadjuvant chemotherapy all patients had modified radical mastectomy or lumpectomy and sentinel lymph node biopsy or axillary node dissection as determined appropriate by the surgeon. Grossly visible residual cancer was measured and representative sections of the cross sectional area were submitted for histopathologic study. When there was not grossly visible residual cancer, the slices of the specimen were radiographed and all areas of radiologically and/or architecturally abnormal tissue were entirely submitted for histopathologic study. Pathologic complete response (pCR) was defined as no residual invasive cancer in the breast or lymph nodes. Residual *in situ* carcinoma (DCIS) without invasive component was also considered pCR. This study was approved by the institutional review boards (IRB) of MDACC and INEN and all patients signed an informed consent for voluntary participation. Clinical characteristics of the patients are presented in Table 1.

Table 1. Characteristics of the Cohorts used for Development and Validation of the Predictor

	Number of Cases (%)	
Female	227	(100%)
Median age (years)	50	(26-75)
Lymph node-negative	62	(27%)
Tumor size		
T1	19	(9%)
T2	131	(58%)
T3	35	(15%)
T4	40	(18%)
Tumor grade		
1	13	(6%)
2	92	(40%)
3	122	(54%)
ER positive	130	(58%)
ER negative	97	(42%)

HER-2 positive	0	(0%)
HER-2 negative	227	(100%)
Adjuvant systemic therapy	Preoperative Paclitaxel-FAC ¹	
Pathologic complete response (pCR)	43	(19%)
Residual Disease (RD)	184	(81%)
None or Minimal Residual Disease (RCB-0/I)	69	(30%)
Moderate Residual Disease (RCB-II)	100	(44%)
Extensive Residual Disease (RCB-III)	58	(26%)

¹Weekly paclitaxel x 12 followed by 3-weekly 5-fluorouracil, doxorubicin, and cyclophosphamide x 4 treatments.

[0088] ***Stratification by Her2-neu and ER status*** - Phenotypic subgroups defined based on the expression or estrogen receptor and amplification of HER2-neu have different response to chemotherapy. FIG. 1 summarizes the stratification of the cases used in this example and the response rates within each subgroup.

[0089] ***RNA extraction and gene expression profiling*** - RNA was extracted from FNA samples using the RNAeasy Kit™ (Qiagen, Valencia CA). The amount and quality of RNA was assessed with DU-640 U.V. Spectrophotometer (Beckman Coulter, Fullerton, CA) and was considered adequate for further analysis if the OD_{260/280} ratio was ≥ 1.8 and the total RNA yield was $\geq 1\mu\text{g}$. cRNA generation and second-strand cDNA synthesis was performed as described previously (Rouzier, 2005; Stec, 2005; Symmans, 2003). No second round amplification was performed. Biotin-labeled cDNA was hybridized to Affymetrix U133A gene chips following the vendor's standard protocols.

[0090] ***Microarray data normalization and data analysis*** - Raw data were generated from Affymetrix chip reader were saved as CEL files. Bioconductor software, which can be found on the World Wide Web at bioconductor.org, was used to generate probe-level intensities and quality measures for each chip. Each chip was normalized using MAS5 (mean=600) using the Bioconductor/R software. Log2-transformed expression values for each probe set were used in subsequent analyses. A reference set of 1322 breast specific (invariant) genes ("housekeeping genes") and their mean expression intensities were established from a reference breast cancer sample database obtained from MD Anderson Cancer Center. For each test sample, a

nonlinear relationship between the intensities of housekeeping genes in the test sample and those of the reference set was determined by fitting a cubic smoothing spline model. This smoothing spline model was then applied to scale the intensities of all probe sets in the array. This normalization scales the probe set intensities in each sample such that the distribution of the housekeeping genes in the test sample matches the distribution in the reference set. All computations are carried out in the software platform R available on the World Wide Web at r-project.org

[0091] ***Methods for building predictors of chemotherapy response based on RCB -***

A critical step when building prediction rules of treatment response or disease state in general from gene expression data is to select a small subset of informative genes that will be used as prognostic features in the predictor. Most predictors employ univariate filtering to rank the candidate genes according to the p-value of a two-sample unequal variance t-test comparing the mean expression values of each gene in the two response classes (*e.g.*, pCR and RD). Univariate filtering methods have the disadvantage that they do not deal well with redundant features (genes that have similar expression profiles) and therefore the resulting predictors tend to be less robust (Lai, 2006). In developing the RCB-based predictor, the inventors used an approach that combines feature selection and model discovery using a multivariate penalized approach called Gradient Directed Regularization developed by Prof. J. Friedman at Stanford University, a description of which can be found on the World Wide Web at stat.stanford.edu/~jhf/ftp/pathlite.pdf. The informative genes are selected through penalization using the maximization of the area under the ROC curve (AUC) as the optimization criterion. Ma and Huang have previously used a similar approach for disease classification (Ma, 2006).

[0092] For predictor discovery and evaluation the inventors followed the rigorous protocol suggested by Wessels *et al.* (Wessels, 2005). The methodology is shown in FIG. 2 and briefly explained below. First, the input dataset is randomly partitioned into a training set and a test set. A 3-fold cross-validation based on Dudoit *et al.* recommendation of a 2:1 split between training and test sets was used (Dudoit, 2002). The training set consisting of 2/3 of the original data is used to develop the predictor. To account for bias in the several data-dependent decisions involved in building the predictor, a 5-fold internal cross-validation is used to select the optimal set of genes

for the predictor and to tune the parameters of the predictor. Details for this process are given below. Since different optimal reporter gene sets might result from the different internal cross-validation folds, the number of times each gene is selected is tracked to provide a measure of its importance or its reliability. The trained predictor is then tested on the 1/5 hold-out part of the training dataset and its performance is evaluated based on the AUC.

[0093] To obtain a less biased estimate of classification performance, the trained predictor is then evaluated on the test set (1/3 of the original data) that was not used in training the predictor. To assess the significance of the predictive performance of the trained predictor, the permutation predictive performance of the predictor was estimated by randomly scrambling the outcome labels in the test dataset. The entire process of randomly splitting the data to a training and a test set was repeated 100 times to obtain the distributions and summary statistics of the performance metrics.

[0094] The method used to identify predictive genes involved comparing samples of patients with good response to the remainder of the patient cohort. First, applying a filter to the gene expression data of all probes on the U133A gene chip, 8150 differentially expressed probe sets were selected that were also consistently expressed in both tissue and cytological samples. Additional filters were subsequently applied to remove those probe sets that did not exhibit adequate variability across samples. For example, genes were removed if expression intensity in at least 50% of the samples did not exceed six, or if the interquartile range (IQR) of expression intensity did not exceed 0.5, or if the difference between the 95th and 5th percentiles of expression intensity did not exceed one. This process left 5686 candidate genes. Finally, the top 1000 probe sets to be used in signature development using the above described algorithm. Gene filtering was based on the regularized t-test for the selected response endpoint such as RCB-0 (same as complete response), or RCB-0/I (very good response).

[0095] The predictor is determined by level of penalization and the choice of a decision threshold. The inventors selected the maximum level of penalization resulting in the smallest signatures that yield significant cross-validated predictor performance – this step determines the signature probe sets and their weights. Then, a decision threshold is selected in order to optimize the predictive values of the

predictor. Evaluation of the predictors was based on the joint confidence interval of the positive predictive value (PPV) and the negative predictive value (NPV) of the predictor at 5% significance level (low 95% confidence limit of $PPV \geq$ baseline response rate & low 95% confidence limit of $NPV \geq 1 -$ baseline response rate).

EXAMPLE 2

Predictor for complete pathologic response (pCR)

[0096] Using the analytical method described earlier to identify genes that differentiate responders from non-responders, RCB-0, which constitutes complete pathologic response, was used as the response endpoint. As FIG. 1 shows, in the cohort of 227 patients, 43 patients (18.9%) showed RCB-0 (complete response) after administration of the full course of T/FAC chemotherapy. Table 2 lists the 64 probes (probe set # on Affymetrix U133A gene chip) and corresponding genes that predict likelihood of achieving RCB-0. The genes are ranked, in decreasing order, according to their predictive power to predict RCB-0 in a patient.

Table 2: Genes used for prediction of RCB-0 in Her2-normal patients after T/FAC chemotherapy

Rank	Probeset ID	Gene	Rank	Probeset ID	Gene
1	219730_at	MED18	33	209644_x_at	CDKN2A
2	206392_s_at	RARRES1	34	221986_s_at	KLHL24
3	218503_at	KIAA1797	35	208651_x_at	CD24
4	215957_at	UBE2D1	36	201555_at	MCM3
5	220281_at	SLC12A1	37	220183_s_at	NUDT6
6	204149_s_at	GSTM4	38	203613_s_at	NDUFB6
7	204825_at	MELK	39	219414_at	CLSTN2
8	204400_at	EFS	40	217640_x_at	C18orf24
9	206315_at	CRLF1	41	208763_s_at	TSC22D3
10	202483_s_at	RANBP1	42	202431_s_at	MYC
11	205234_at	SLC16A4	43	208063_s_at	CAPN9
12	212690_at	DDHD2	44	204750_s_at	DSC2
13	215074_at	MYO1B	45	215943_at	KIAA1661
14	205229_s_at	COCH	46	209070_s_at	RGS5
15	202014_at	PPP1R15A	47	206686_at	PDK1
16	213100_at	<NA>	48	215108_x_at	TNRC9
17	209985_s_at	ASCL1	49	203071_at	SEMA3B
18	209035_at	MDK	50	204012_s_at	LCMT2
19	206373_at	ZIC1	51	217867_x_at	BACE2
20	208022_s_at	CDC14B	52	205683_x_at	TPSAB1
21	221276_s_at	SYNC1	53	220935_s_at	CDK5RAP2

22	222031_at	LOC389906	54	204107_at	NFYA
23	219051_x_at	METRNL	55	215034_s_at	TM4SF1
24	210084_x_at	TPSAB1	56	205452_at	PIGB
25	206509_at	PIP	57	212141_at	MCM4
26	203851_at	IGFBP6	58	211466_at	NFIB
27	216977_x_at	SNRPA1	59	206194_at	HOXC4
28	215432_at	ACSM1	60	220238_s_at	KLHL7
29	218902_at	NOTCH1	61	205347_s_at	TMSL8
30	202293_at	STAG1	62	219663_s_at	TMEM121
31	200934_at	DEK	63	205350_at	CRABP1
32	202946_s_at	BTBD3	64	213345_at	NFATC4

[0097] Using this gene list, a predictor was developed to predict a “complete responder” within the same patient cohort (see Patients and Methods) using a decision threshold of 4. A 3-fold cross validation (B=100 with response-based random splits of data) was conducted to assess performance within the dataset. Table 3 summarizes the predictor performance.

Table 3: Cross-validated performance of pCR-based response predictor

	Mean	95% confidence interval	
		Lower	Upper
Accuracy	84.5%	74.5%	91.7%
Sensitivity	70.7%	42.1%	90.3%
Specificity	87.7%	77.1%	94.5%
Positive predictive value (PPV)	58.1%	0.327*	80.2%
Negative predictive value (NPV)	92.9%	0.831*	97.8%

* significant at 5% level

[0098] The performance of the predictor was also evaluated in a cohort of Her2-positive patients (n=33) to assess how well the stratification based on Her2 marker status improves performance of the predictor. Fig. 3 shows the comparison of the predictor classification of patients in the Her2-normal and Her2-positive cohorts. The predictor is uninformative in the latter cohort indicating its specific value in patients whose Her2 receptor status is normal.

EXAMPLE 3**Predictor of response using stratification of estrogen receptor (ER) status**

[0099] The combined endpoints of pCR or RCB-0, and RCB-I, which constitutes near-pCR, were used. In this analysis, the inventors further stratified these patients according to the status of the estrogen receptor (ER) as shown at the bottom of FIG. 1. Of the total 227 patients, 130 patients (57.2%) were ER-positive and the remainder was ER-negative. The proportion of excellent response, indicated by the sum of RCB-0 and RCB-I cases, was 16.2% in ER-positive patients and 49.5% in ER-negative patients. The difference in the baseline response indicates that ER status, and accounting for it in predictor development, may be used to achieve optimal diagnostic performance.

[00100] Using each of the ER-stratified subsets (130 ER-positive and 97 ER-negative), gene sets were separately identified for their ability to discriminate between good responders (RCB-0/I) versus the rest. The methodology used to identify genes in each of the subsets was the same as described before. A gene set of 89 probes was established for ER-positive patients, and a gene set of 116 probes was established for ER-negative patients. These are listed in Table 4.

Table 4: Genes used for prediction of RCB-0/I in ER-stratified patient subsets

ER Positive

Rank	Probeset	Gene		Probeset	Gene
1	209832_s_at	CDT1	46	200867_at	ZNF313
2	212668_at	SMURF1	47	34187_at	RBMS2
3	216200_at	PLEKHM1	48	201986_at	THRAP1
4	203352_at	ORC4L	49	221585_at	CACNG4
5	210702_s_at	PTGIS	50	221040_at	CAPN10
6	211592_s_at	CACNA1C	51	218277_s_at	DHX40
7	207109_at	POU2F3	52	200614_at	CLTC
8	206591_at	RAG1	53	218103_at	FTSJ3
9	209621_s_at	PDLIM3	54	218098_at	ARFGEF2
10	217032_at	FOXD4L1	55	213068_at	DPT
11	218323_at	RHOT1	56	201315_x_at	IFITM2
12	AFFX-r2-Bs-lys-3_at	<NA>	57	218925_s_at	C11orf1
13	213299_at	ZBTB7A	58	215957_at	UBE2D1
14	220945_x_at	MANSC1	59	220652_at	KIF24
15	210611_s_at	DTNA	60	219908_at	DKK2
16	211678_s_at	ZNF313	61	214292_at	ITGB4
17	62987_r_at	CACNG4	62	217582_at	KCNIP4
18	204671_s_at	ANKRD6	63	213699_s_at	YWHAQ
19	213946_s_at	OBSL1	64	206066_s_at	RAD51C

20	213439_x_at	RPIP8	65	203684_s_at	BCL2
21	203906_at	IQSEC1	66	205522_at	HOXD4
22	208247_at	C3orf51	67	209234_at	KIF1B
23	212353_at	SULF1	68	201149_s_at	TIMP3
24	206995_x_at	SCARF1	69	205421_at	SLC22A3
25	209821_at	IL33	70	213868_s_at	DHRS7
26	219103_at	DDEFL1	71	219046_s_at	PKNOX2
27	205145_s_at	MYL5	72	211341_at	POU4F1
28	211522_s_at	GNRHR	73	203281_s_at	UBE1L
29	203001_s_at	STMN2	74	221522_at	ANKRD27
30	217306_at	ZNF131	75	206068_s_at	ACADL
31	213510_x_at	FAM106A	76	207425_s_at	SEPT9
32	202762_at	ROCK2	77	219417_s_at	C17orf59
33	201002_s_at	Kua-UEV	78	202335_s_at	UBE2B
34	209204_at	LMO4	79	206877_at	MXD1
35	216795_at	<NA>	80	207491_at	MOGAT2
36	221198_at	SCT	81	205838_at	GYPA
37	205222_at	EHHADH	82	211936_at	HSPA5
38	1294_at	UBE1L	83	207773_x_at	CYP3A43
39	220607_x_at	TH1L	84	39548_at	NPAS2
40	216504_s_at	SLC39A8	85	206331_at	CALCRL
41	220022_at	ZNF334	86	207623_at	ABCF2
42	201987_at	THRAP1	87	218918_at	MAN1C1
43	212474_at	KIAA0241	88	212816_s_at	CBS
44	217200_x_at	CYB561	89	217564_s_at	CPS1
45	218732_at	PTRH2			

ER Negative

Rank	Probeset	Gene		Probeset	Gene
1	201910_at	FARP1	59	217678_at	SLC7A11
2	217254_s_at	EPO	60	203954_x_at	CLDN3
3	212294_at	GNG12	61	207695_s_at	IGSF1
4	205700_at	HSD17B6	62	211901_s_at	PDE4A
5	221311_x_at	LYRM2	63	206508_at	CD70
6	214479_at	GFRA3	64	207371_at	<NA>
7	220154_at	DST	65	221031_s_at	APOLD1
8	206432_at	HAS2	66	215818_at	NUDT7
9	210096_at	CYP4B1	67	203792_x_at	PCGF2
10	206373_at	ZIC1	68	210160_at	PAFAH1B2
11	213793_s_at	HOMER1	69	216962_at	C1QBP
12	208920_at	SRI	70	203633_at	CPT1A
13	221835_at	DTX3	71	220820_at	<NA>
14	205617_at	PRRG2	72	219071_x_at	C8orf30A
15	203782_s_at	POLRMT	73	210391_at	NR6A1
16	202865_at	DNAJB12	74	208222_at	ACVR1B
17	220358_at	SNFT	75	200838_at	CTSB
18	213633_at	SH3BP1	76	213019_at	RANBP6
19	211990_at	HLA-DPA1	77	215733_x_at	CTAG2
20	208481_at	ASB4	78	204156_at	KIAA0999
21	220298_s_at	SPATA6	79	211792_s_at	CDKN2C
22	211386_at	MGC12488	80	205301_s_at	OGG1
23	206952_at	G6PC	81	218029_at	FAM65A
24	210678_s_at	AGPAT2	82	219356_s_at	CHMP5
25	220183_s_at	NUDT6	83	204844_at	ENPEP

26	215718_s_at	PHF3	84	201826_s_at	SCCPDH
27	212149_at	KIAA0143	85	206944_at	HTR6
28	221122_at	HRASLS2	86	221629_x_at	C8orf30A
29	205302_at	IGFBP1	87	209533_s_at	PLAA
30	214776_x_at	XYLB	88	208240_s_at	<NA>
31	217640_x_at	C18orf24	89	205675_at	MTTP
32	204544_at	HPS5	90	210680_s_at	MASP1
33	210263_at	KCNF1	91	210221_at	CHRNA3
34	208075_s_at	CCL7	92	217695_x_at	PELI1
35	201779_s_at	RNF13	93	217319_x_at	CYP4A11
36	221288_at	GPR22	94	214109_at	LRBA
37	202463_s_at	MBD3	95	213081_at	ZBTB22
38	208863_s_at	SFRS1	96	220737_at	RPS6KA6
39	220400_at	VPS13B	97	220595_at	PDZRN4
40	205246_at	PEX13	98	207680_x_at	PAX3
41	203286_at	RNF44	99	207940_x_at	CNR1
42	201801_s_at	SLC29A1	100	206655_s_at	GP1BB
43	211149_at	UTY	101	201404_x_at	PSMB2
44	212220_at	PSME4	102	205345_at	BARD1
45	217111_at	AMACR	103	204400_at	EFS
46	207130_at	ZMYND8	104	216703_at	<NA>
47	214074_s_at	CTTN	105	211430_s_at	IGHG3
48	215012_at	ZNF451	106	221276_s_at	SYNC1
49	203736_s_at	PPFIBP1	107	218651_s_at	LARP6
50	205239_at	AREG	108	216685_s_at	MTAP
51	221244_s_at	PDPK1	109	213407_at	PHLPPL
52	210138_at	RGS20	110	202508_s_at	SNAP25
53	217382_at	CLIC1	111	203889_at	SCG5
54	221986_s_at	KLHL24	112	209772_s_at	CD24
55	208926_at	NEU1	113	215172_at	PTPN20B
56	211425_x_at	SSX2	114	214413_at	TAT
57	203725_at	GADD45A	115	203849_s_at	KIF1A
58	206568_at	TNP1	116	215833_s_at	SPPL2B

[00101] Using these gene lists, a composite predictor was developed to predict a “good responder” within the total patient dataset. A decision threshold of 2 was used for the ER-positive response predictor and a decision threshold of 0 for the ER-negative predictor. The results of the 3-fold cross validation analyses (B=100 with response-based random splits of data) of each of the predictors on their respective datasets are presented in Table 5. Figure 4 shows the performance of the predictors in predicting good response in the total patient dataset.

[00102] The performance of the predictor was evaluated in a cohort of Her2-positive patients (n=33) to assess how well the stratification based on Her2 marker status improves performance of the predictor. FIG. 4 shows the comparison of the predictor classification of patients in the Her2-normal, FIG. 4A (n=227) and Her2-positive,

FIG. 4B (n=33) cohorts. The predictor is not informative in the latter cohort indicating its specific value in patients whose Her2 receptor status is normal.

Table 5: Cross-validated performance of ER-stratified RCB-0/I predictors

ER-positive predictor

	Mean	95% confidence interval	
		Lower	Upper
Accuracy	80.5%	66.1%	90.6%
Sensitivity	64.1%	25.7%	91.4%
Specificity	83.7%	68.2%	93.4%
Positive predictive value (PPV)	44.2%	15.9%	75.4%
Negative predictive value (NPV)	92.4%	78.1%	98.4%

ER-negative predictor

	Mean	95% confidence interval	
		Lower	Upper
Accuracy	77.5%	59.5%	90.1%
Sensitivity	78.0%	51.2%	93.5%
Specificity	77.1%	50.2%	93.2%
Positive predictive value (PPV)	77.8%	50.5%*	93.7%
Negative predictive value (NPV)	78.4%	51.0%*	93.9%

* significant at 5% level

[00103] FIG. 5 shows the survival outcome of patients defined by distant relapse-free survival (DRFS) over a period of about 7 years. These patients have undergone surgery where it was considered appropriate and the ER-positive patients received hormonal therapy (tamoxifen) for 5 years after the surgery. ER-negative patients did not receive any treatment post-surgery. The plot shows that predicted high responders to T/FAC chemotherapy (FIG. 5) show a high proportion of patients free of distant relapse (~90%) whereas the remainder show considerably lower relapse-free survival among the patients (~60% in about 7 years). The two curves are statistically distinct ($p < 0.001$) indicating that the composite predictor of good response among patients

can discriminate patients effectively for survival-related response with high statistical significance.

[00104] FIG. 6 shows the survival outcome as distant relapse-free survival (DRFS) in ER-stratified patients over a period of about 7 years. These patients are the same as those shown in FIG. 5 but stratified for ER status. All patients have undergone surgery where it was considered appropriate and the ER-positive patients received hormonal therapy (tamoxifen) for 5 years after the surgery. ER-negative patients did not receive any treatment post-surgery. The analysis of performance separately in ER-stratified patients is important to examine and account for heterogeneity of tumors and the corresponding differences in response to therapy. The plots show that, in both groups, predicted high responders to T/FAC chemotherapy (FIG. 6) show a high proportion of patients free of distant relapse (>85% in ER-positive patients and ~90% in ER-negative patients) whereas the remainder in both groups show considerably lower relapse-free survival among the patients). The numbers on the plots are the percentage of patients within the selected ER-stratified category that fall either into responders or non-responders.

EXAMPLE 4

Predictor of response using residual cancer burden (RCB) and stratification of estrogen receptor (ER) status

[00105] The combined endpoints of RCB-0, which constitutes complete pathologic response, and RCB-I, which constitutes minimal residual disease, were used as in Example 3. The inclusion of RCB-I allows extending the response and its favorable outcome to potentially better predict patients who have the best benefit of therapy. In this analysis, the inventors further stratified these patients according to the status of the estrogen receptor (ER) as shown at the bottom of FIG. 1. Of the total 227 patients, 130 patients (57.2%) were ER-positive and the remainder was ER-negative. The proportion of excellent response, indicated by the sum of RCB-0 and RCB-I cases, was 16.2% in ER-positive patients and 49.5% in ER-negative patients. The difference in baseline response rate indicates that ER status, and accounting for it in

predictor development, may be an important requirement to achieve optimal diagnostic performance.

[00106] Compared to Example 3, a difference in analysis methodology was employed in this example because different types of biological specimens might contain different numbers of tumor cells compared to stromal cells or cells from other contaminating organs (Symmans et al, 2003). Steps were taken, therefore, to improve the robustness of the predictors with respect to their applicability to different sample types. First, a reference set of 1322 of breast cancer specific genes were identified from a set of core biopsies, fine needle aspirations and lymph node biopsies. A reference distribution for these breast cancer specific genes was established from a database of breast cancer fine needle aspiration samples. This reference distribution was then used to scale (normalize) microarray profiles generated from breast tumor biopsies. After scaling, individual probe sets were evaluated for concordance of expression in matched pairs of core biopsy and fine need aspiration breast cancer samples. Probe sets that exhibited concordance bias of more than 5% were removed from the candidate pool for predictor development. This resulted in a response predictor that yields considerably more concordant results when applied to core biopsies or fine need aspiration samples from the same tumor.

[00107] Using each of the ER-stratified subsets (130 ER-positive and 97 ER-negative), gene sets were separately identified for their ability to discriminate between good responders (RCB-0/I) versus the rest. The methodology used to identify genes in each of the subsets was the same as described before. A total gene set of 107 probes was established for ER-positive patients, and a total gene set of 135 probes was established for ER-negative patients. These are listed in Table 4. The genes are ranked in each Table, in decreasing order, according to their ability to predict RCB-0/I in a patient.

Table 6: Genes used for prediction of RCB-0/I in ER-stratified patient subsets

ER-Positive Predictor

Rank	Probeset	Gene
1	210589_s_at	GBA
2	211678_s_at	ZNF313
3	201986_at	THRAP1
4	201649_at	UBE2L6

5	218098_at	ARFGEF2
6	212426_s_at	YWHAQ
7	211936_at	HSPA5
8	202124_s_at	TRAK2
9	213946_s_at	OBSL1
10	214523_at	CEBPE
11	32069_at	N4BP1
12	213434_at	STX2
13	207109_at	POU2F3
14	212816_s_at	CBS
15	208247_at	C3orf51
16	203352_at	ORC4L
17	34187_at	RBMS2
18	211937_at	EIF4B
19	211817_s_at	KCNJ5
20	219327_s_at	GPRC5C
21	214154_s_at	PKP2
22	221820_s_at	MYST1
23	208818_s_at	COMT
24	206457_s_at	DIO1
25	221279_at	GDAP1
26	216504_s_at	SLC39A8
27	214751_at	ZNF468
28	206415_at	TLL1
29	222270_at	SMEK2
30	206706_at	NTF3
31	220938_s_at	GMEB1
32	200614_at	CLTC
33	204215_at	C7orf23
34	213573_at	KPNB1
35	222272_x_at	SCIN
36	211018_at	LSS
37	204913_s_at	SOX11
38	203906_at	IQSEC1
39	218732_at	PTRH2
40	220945_x_at	MANSC1
41	205665_at	TSPAN9
42	210528_at	MR1
43	212710_at	CAMSAP1
44	210955_at	CASP10
45	218103_at	FTSJ3
46	210527_x_at	TUBA3C
47	211786_at	TNFRSF9
48	202879_s_at	PSCD1
49	200651_at	GNB2L1
50	219255_x_at	IL17RB
51	221522_at	ANKRD27
52	211249_at	GPR68
53	204915_s_at	SOX11
54	214022_s_at	IFITM1

55	208213_s_at	KCNAB1
56	218398_at	MRPS30
57	210300_at	REM1
58	220842_at	AHI1
59	215901_at	NA
60	202464_s_at	PFKFB3
61	202335_s_at	UBE2B
62	201002_s_at	Kua-UEV
63	205534_at	PCDH7
64	220297_at	BTBD7
65	213918_s_at	NIPBL
66	218592_s_at	CECR5
67	205663_at	PCBP3
68	201922_at	TINP1
69	221877_at	NA
70	207729_at	CDH9
71	220652_at	KIF24
72	213037_x_at	STAU1
73	201288_at	ARHGDIB
74	214105_at	SOCS3
75	201112_s_at	CSE1L
76	222231_s_at	LRRC59
77	210867_at	CNOT4
78	203534_at	LSM1
79	209470_s_at	GPM6A
80	222150_s_at	LOC54103
81	214796_at	KIAA1456
82	206253_at	DLG2
83	217697_at	FYN
84	214306_at	OPA1
85	222063_s_at	CDS1
86	218699_at	RAB7L1
87	209988_s_at	ASCL1
88	202222_s_at	DES
89	208704_x_at	APLP2
90	220029_at	ELOVL2
91	220931_at	MGC5590
92	210389_x_at	TUBD1
93	205987_at	CD1C
94	207739_s_at	GAGE2
95	201236_s_at	BTG2
96	201998_at	ST6GAL1
97	215407_s_at	ASTN2
98	210618_at	RAP1GAP
99	213401_s_at	TBL1X
100	217651_at	NA
101	206068_s_at	ACADL
102	205751_at	SH3GL2
103	213524_s_at	G0S2
104	211068_x_at	FAM21C

105	37547_at	BBS9
106	209859_at	TRIM9
107	207101_at	VAMP1

ER-Negative Predictor

Rank	Probeset	Gene
1	211425_x_at	SSX2
2	204246_s_at	DCTN3
3	215227_x_at	ACP1
4	203538_at	CAMLG
5	219260_s_at	C17orf81
6	206508_at	CD70
7	212971_at	CARS
8	208107_s_at	LOC81691
9	217254_s_at	EPO
10	213117_at	KLHL9
11	210096_at	CYP4B1
12	213091_at	CRTC1
13	211192_s_at	CD84
14	219326_s_at	B3GNT2
15	221835_at	DTX3
16	220358_at	SNFT
17	203566_s_at	AGL
18	204617_s_at	ACD
19	214681_at	GK
20	221830_at	RAP2A
21	220183_s_at	NUDT6
22	204568_at	KIAA0831
23	220844_at	TCEB3B
24	205440_s_at	NPY1R
25	213793_s_at	HOMER1
26	204290_s_at	ALDH6A1
27	203106_s_at	VPS41
28	206002_at	GPR64
29	211924_s_at	PLAUR
30	214160_at	CDK6
31	211386_at	MGC12488
32	206239_s_at	SPINK1
33	211400_at	FGFR2
34	216703_at	NA
35	210625_s_at	AKAP1
36	210263_at	KCNF1
37	217951_s_at	PHF3
38	214532_x_at	POU5F1P1
39	205239_at	AREG
40	213078_x_at	AGPAT7
41	207930_at	LCN1
42	217952_x_at	PHF3
43	210138_at	RGS20

44	213408_s_at	LOC728233
45	217286_s_at	NDRG3
46	205675_at	MTTP
47	219051_x_at	METRN
48	205684_s_at	DENND4C
49	213045_at	MAST3
50	214172_x_at	RYK
51	214131_at	CYorf15B
52	206410_at	NR0B2
53	210680_s_at	MASP1
54	213359_at	HNRPD
55	205246_at	PEX13
56	209644_x_at	CDKN2A
57	214840_at	TOM1L2
58	207236_at	ZNF345
59	211118_x_at	ESR2
60	213110_s_at	COL4A5
61	217840_at	DDX41
62	202588_at	AK1
63	202463_s_at	MBD3
64	206938_at	SRD5A2
65	207861_at	CCL22
66	222141_at	KLHL22
67	203904_x_at	CD82
68	214444_s_at	PVR
69	205302_at	IGFBP1
70	215034_s_at	TM4SF1
71	208895_s_at	DDX18
72	206278_at	PTAFR
73	209387_s_at	TM4SF1
74	214074_s_at	CTTN
75	204078_at	SC65
76	222266_at	C19orf2
77	216685_s_at	MTAP
78	207775_at	MGC4859
79	213015_at	NA
80	211883_x_at	CEACAM1
81	210639_s_at	ATG5
82	209277_at	TFPI2
83	218699_at	RAB7L1
84	46947_at	GNL3L
85	211613_s_at	GPD2
86	203138_at	HAT1
87	205973_at	FEZ1
88	200934_at	DEK
89	202583_s_at	RANBP9
90	204751_x_at	DSC2
91	202535_at	FADD
92	205079_s_at	MPDZ
93	208027_s_at	TLL2

94	215685_s_at	DLX2
95	200926_at	RPS23
96	210601_at	CDH6
97	219597_s_at	DUOX1
98	210471_s_at	KCNAB1
99	209386_at	TM4SF1
100	217681_at	WNT7B
101	220400_at	VPS13B
102	200711_s_at	TCEB1P
103	205774_at	F12
104	200970_s_at	SERP1
105	218117_at	RBX1
106	203831_at	R3HDM2
107	204600_at	EPHB3
108	220046_s_at	CCNL1
109	209863_s_at	TP73L
110	213157_s_at	KIAA0523
111	204990_s_at	ITGB4
112	213328_at	NEK1
113	213245_at	ADCY1
114	37950_at	PREP
115	203653_s_at	COIL
116	220425_x_at	ROPN1
117	210221_at	CHRNA3
118	209710_at	GATA2
119	210319_x_at	MSX2
120	208222_at	ACVR1B
121	208595_s_at	MBD1
122	217248_s_at	SLC7A8
123	208244_at	BMP3
124	215958_at	RAB5A
125	207860_at	NCR1
126	216537_s_at	SIGLEC7
127	212045_at	GLG1
128	214859_at	FSTL4
129	209069_s_at	H3F3B
130	213959_s_at	KIAA1005
131	217678_at	SLC7A11
132	205952_at	KCNK3
133	201207_at	TNFAIP1
134	213930_at	NA
135	208086_s_at	DMD

[00108] Using these gene lists, a composite predictor was developed to predict a “very good responder” within the total patient dataset. A decision threshold of 3 was used for the ER-positive response predictor, and a decision threshold of 0 for the ER-negative predictor. The results of the 3-fold cross validation analyses (B=100 with response-based random splits of data) of each of the predictors on their respective

datasets are presented in Table 7. Within the predictive algorithm, ER status of a patient was determined by comparing the expression level of probe set “205225_at” for ESR1 gene with the pre-established cutoff value of 10.58.

Table 7: Cross-validated performance of ER-stratified RCB-0/I predictors

ER-positive predictor

	Mean	95% confidence interval	
		Lower	Upper
Accuracy	86.9%	73.4%	95.1%
Sensitivity	45.2%	12.8%	81.5%
Specificity	95.0%	82.5%	99.1%
Positive predictive value (PPV)	65.5%	18.4%	93.8%
Negative predictive value (NPV)	90.1%	76.1%	97.2%

ER-negative predictor

	Mean	95% confidence interval	
		Lower	Upper
Accuracy	77.2%	59.4%	89.6%
Sensitivity	75.6%	49.0%	92.3%
Specificity	78.7%	52.5%	93.8%
Positive predictive value (PPV)	78.1%	50.7%*	93.8%
Negative predictive value (NPV)	77.2%	50.9%*	93.1%

* significant at 5% level

[00109] FIG. 7 shows the composite resubstitution performance of the predictors in predicting excellent response (RCB—0/I) in the total patient dataset. The different thresholds employed for ER-positive and ER-negative patients are also shown in the figure. As shown by FIG. 7, the response output by the predictor of all cases in this development cohort was reported correctly. However, in general, measurement of the resubstitution performance reflects an optimistic value of prediction efficiency whereas the predictor efficiency in an independent patient cohort may be somewhat lower.

[00110] FIG. 8 shows the survival outcome of patients defined by distant relapse-free survival (DRFS) over a period of about 7 years. These patients have undergone

surgery where it was considered appropriate and the ER-positive patients received hormonal therapy (tamoxifen) for 5 years after the surgery. ER-negative patients did not receive any treatment post-surgery. The plot shows that predicted high responders to T/FAC chemotherapy (FIG. 8) show a high proportion of patients free of distant relapse (~90% DRFS) whereas the remainder (FIG. 8) show considerably lower relapse-free survival among the patients (~60% in about 7 years). The numbers shown represent the percentage of total patients who are in the respective category. The two curves are statistically distinct ($p < 0.001$) indicating that the composite predictor of good response among patients can discriminate patients effectively for survival-related response with high statistical significance.

[00111] FIG. 9 shows the survival outcome as distant relapse-free survival (DRFS) in ER-stratified patients over a period of about 7 years. These patients are the same as those shown in FIG. 8 but stratified for ER status. All patients have undergone surgery where it was considered appropriate and the ER-positive patients received hormonal therapy (tamoxifen) for 5 years after the surgery. ER-negative patients did not receive any treatment post-surgery. The analysis of performance separately in ER-stratified patients is important to examine and account for heterogeneity of tumors and the corresponding differences in response to therapy. The plots show that, in both groups, predicted high responders to T/FAC chemotherapy (FIG. 9) show a high proportion of patients free of distant relapse (>90% in ER-positive patients and ~85% in ER-negative patients) whereas the remainder in both groups show considerably lower relapse-free survival among the patients). The numbers shown represent the percentage of total patients who are in the respective category. The two curves are statistically distinct ($p = 0.03$ and $p < 0.001$, respectively) in both groups indicating that the composite predictor of good response among patients can discriminate patients effectively even within specific ER-stratified subgroups.

[00112] The analysis in FIG. 9 represents a significant advance over conventional techniques of therapy selection because of the predictor's ability to account for tumor heterogeneity and its inherent impact of response outcomes from therapy. ER-positive tumors, in general, have a better overall prognosis than ER-negative tumors, as evident from comparing the average baselines of DRFS between the left (ER-positive) and right (ER-negative) panels of FIG. 9. In ER-positive tumors, the

predictor exhibits strong predictive power in separating good responders (17.6% of patients) from remainder who are poor responders, where good response translates to excellent survival post-therapy. More significantly, in ER-negative and Her2-negative tumors (shown at right in FIG. 9), which are considered difficult to treat and have inherently poor prognosis, the predictor displays excellent predictive power in identifying very good responders to TFAC chemotherapy (47.9% of patients) who have >85% relapse-free survival after 5 years. In contrast, the remainder of this ER-negative, Her2-negative group (52.1% of patients) show an alarmingly low ~30% relapse-free survival in 5 years. The ability to distinguish likely survival outcome resulting from standard chemotherapy in these patient cohorts allows physicians not only to provide the benefit of a specific chemotherapy selection to good responders but also to facilitate alternative treatments and clinical trials to those who are poor responders and face a high chance of cancer recurrence with standard therapy.

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CLAIMS

1. A method of evaluating responsiveness of a tumor to therapy in a cancer patient comprising the steps of:
 - (a) obtaining a sample of a tumor from a cancer patient
 - (b) evaluating gene expression levels in a patient sample comprising cancer cells or an RNA sample isolated from such a patient sample, wherein a plurality of genes to be evaluated are the 64 genes listed in Table 2, or the 89 ER positive genes listed in Table 4, or the 116 ER negative genes listed in Table 4, or at least 62 of the ER positive genes listed in Table 6, at least the 64 of the ER negative genes listed in Table 6;
 - (c) calculating a predictor score using a predetermined transcriptional profile of the selected genes; and
 - (d) assessing the likelihood of a therapeutic outcome using the predictor score.
2. The method of claim 1, further comprising identifying a cancer patient with a disease state classified as a residual disease state prior to evaluation.
3. The method of claim 1, wherein the sample is obtained by aspiration, biopsy or surgical resection.
4. The method of claim 1, wherein the therapeutic outcome of cancer therapy is distant relapse-free survival (DRFS).
5. The method of claim 1, wherein the plurality of genes to be evaluated are at least the first 62 ER negative genes of Table 6.
6. The method of claim 1, wherein the plurality of genes to be evaluated are all ER negative genes of Table 6.
7. The method of claim 1, wherein the plurality of genes to be evaluated are at least the first 64 ER positive genes of Table 6.

8. The method of claim 1, wherein the plurality of genes to be evaluated are all ER positive genes of Table 6.
9. The method of claim 1, wherein the plurality of genes to be evaluated are a combination of all ER positive and ER negative genes of Table 6.
10. The method of claim 1, further comprising determining Her2-neu and/or estrogen receptor status of the patient sample.
11. The method of claim 1, wherein the predictor score includes evaluation of tumor size, cellularity of tumor bed, and/or nodal burden.
12. The method of claim 1, further comprising providing a therapy recommendation depending on the predictor score.
13. The method of claim 12, wherein the therapy is a combination of one or more cancer therapy.
14. The method of claim 13, wherein the therapy is chemotherapy.
15. The method of claim 14, wherein the chemotherapy comprises a taxane chemotherapy.
16. The method of claim 15, wherein the chemotherapy is T/FAC therapy.
17. The method of claim 1, wherein selecting the genes to be included in the transcriptional profile comprises the steps of:
 - (a) obtaining data associated with a plurality of breast cancer patients comprising measuring expression levels of a plurality of genes in samples from the patients;
 - (b) partitioning the data into a first and second dataset;
 - (c) evaluating the data and identifying data associated with a particular treatment outcome;
 - (d) selecting a set of genes whose expression levels are indicative of therapeutic outcome

18. The method of claim 17, wherein the selection of genes to be included in the transcriptional profile includes evaluation of survival of the patient population sampled for all or part of the reference population of tumor samples.
19. The method of claim 18, wherein the method includes evaluation of distant relapse-free survival (DRFS) of the patient population.
20. The method of claim 1, wherein assessing the expression of the one or more markers comprises detecting mRNA of the one or more markers.
21. The method of claim 20, wherein the detection comprises microarray analysis.
22. The method of claim 20, wherein the detection comprises PCR.
23. The method of claim 20, wherein the detection comprises in situ hybridization.
24. The method of claim 1, wherein assessing the expression of the one or more markers comprises detecting the protein encoded by one or more markers.
25. The method of claim 24, wherein detecting the protein is by immunohistochemistry.
26. A kit to determine responsiveness of a cancer comprising:
 - (a) reagents for determining expression levels of a plurality of genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof; and
 - (b) software encoding an algorithm for calculating a predictor score based on the analysis of the gene expression levels.
27. A system for providing assessment of a sample relative to a gene expression index, the system comprising:
 - (a) an application server comprising an input manager to receive expression data from a user for a plurality of

- genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof obtained from a patient sample or an RNA sample from such patient sample; and
- (b) a network server comprising an output manager constructed and arranged to provide an assessment to the user.
28. A computer readable medium having software modules for performing the method of claim 1 comprising the acts of:
- (a) comparing gene expression data obtained from a patient sample for a plurality of genes selected from Table 2 or ER positive genes of Table 4 or ER negative genes of Table 4 or ER positive genes of Table 6 or ER negative genes of Table 6 or combinations thereof with a reference; and
- (b) providing a predictor score to a physician for use in determining an appropriate therapeutic regimen for a patient.
29. A computer system, having a processor, memory, external data storage, input/output mechanisms, a display, for performing the method of claim 1, comprising:
- (a) a database;
- (b) logic mechanisms in the computer for generating the transcriptional profile; and
- (c) a comparing mechanism in the computer for comparing the gene expression reference to expression data from a patient sample or an RNA sample from such a patient sample to calculate a predictor score.
30. An internet accessible portal for providing biological information constructed and arranged to execute a computer-implemented method of claim 1 for providing:

- (a) a comparison of gene expression data of a plurality of genes of claim 1 in a patient sample with a transcriptional profile; and
- (b) providing a predictor score to a physician for use in determining an appropriate therapeutic regime for a patient.

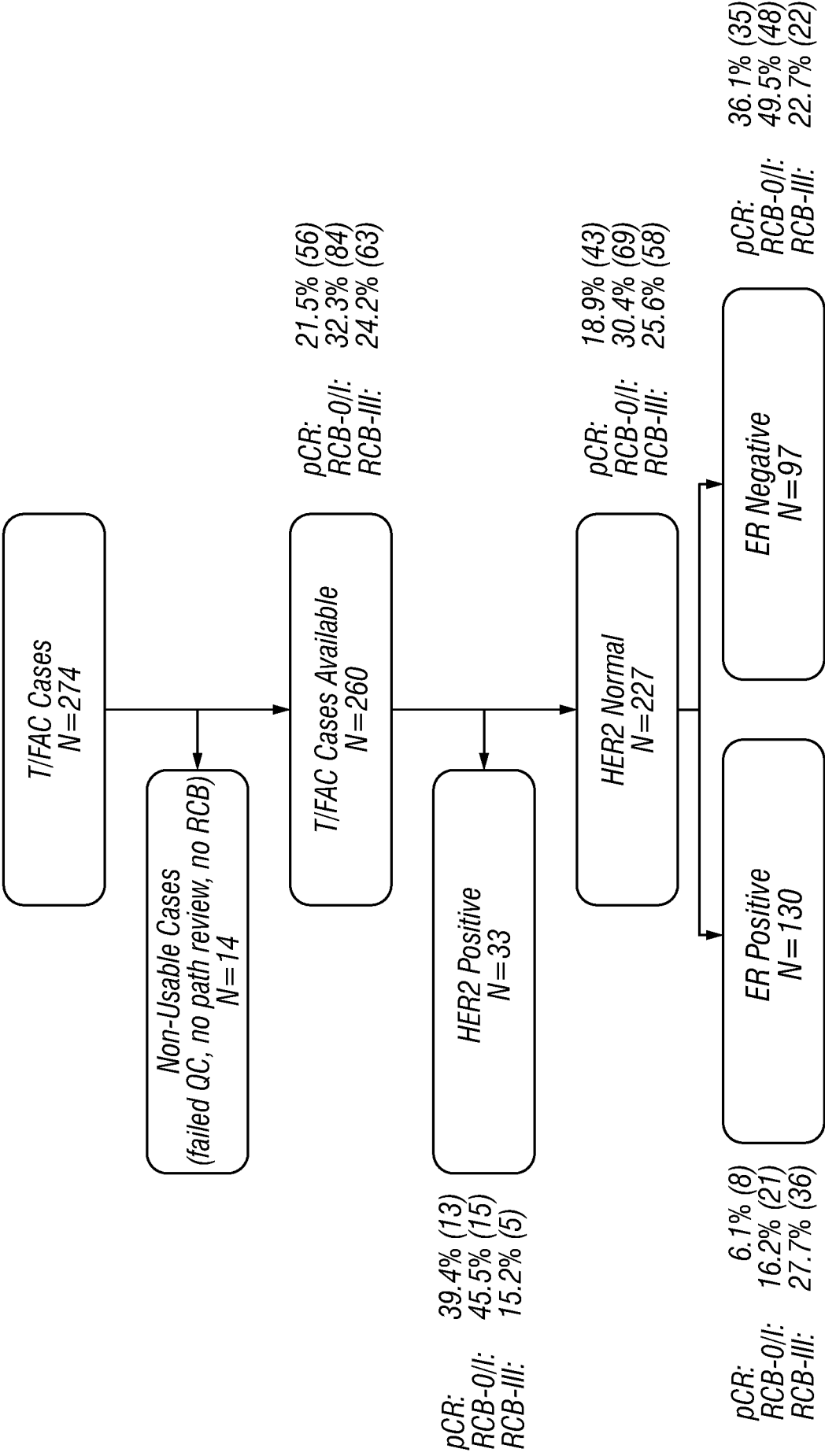


FIG. 1

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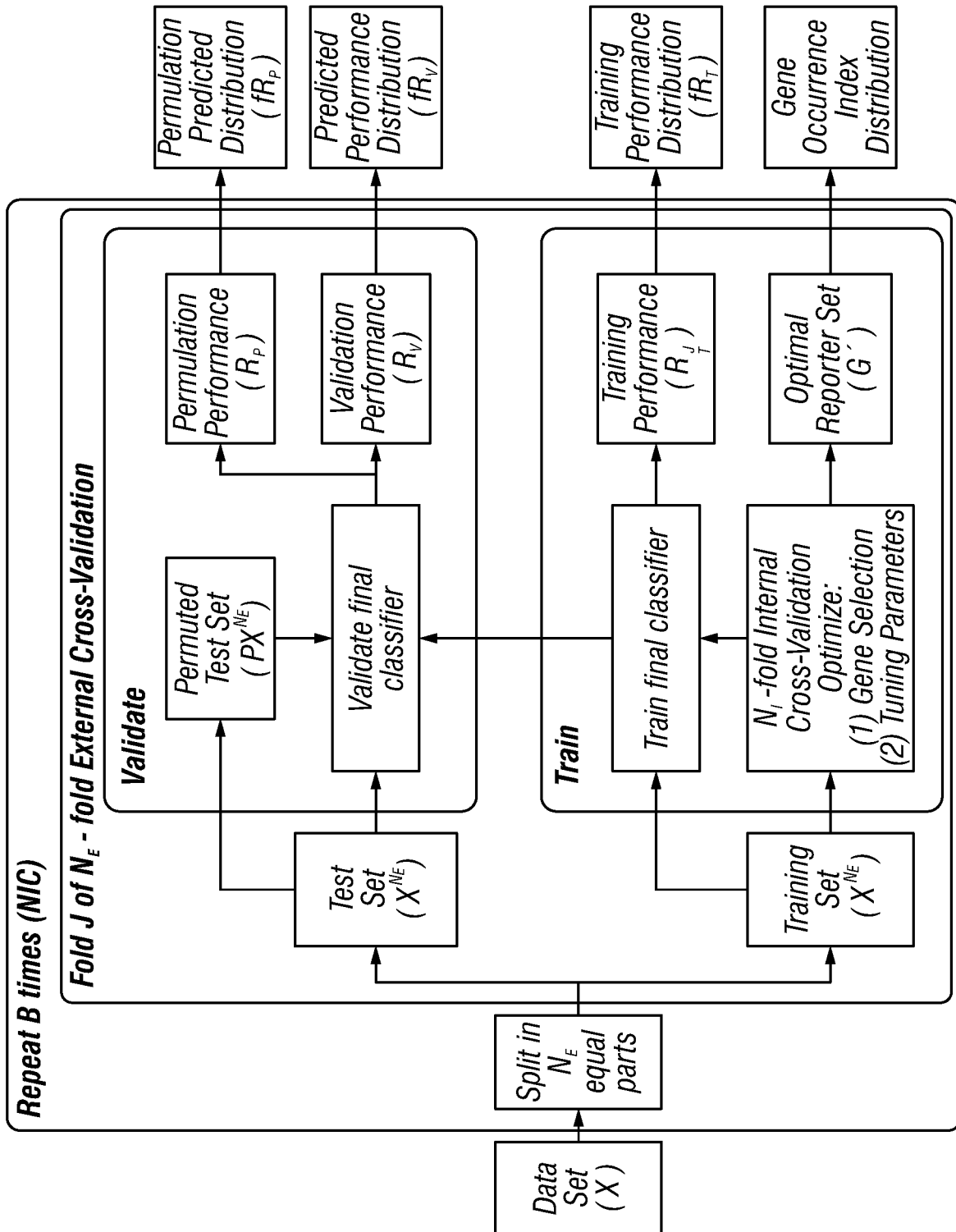


FIG. 2

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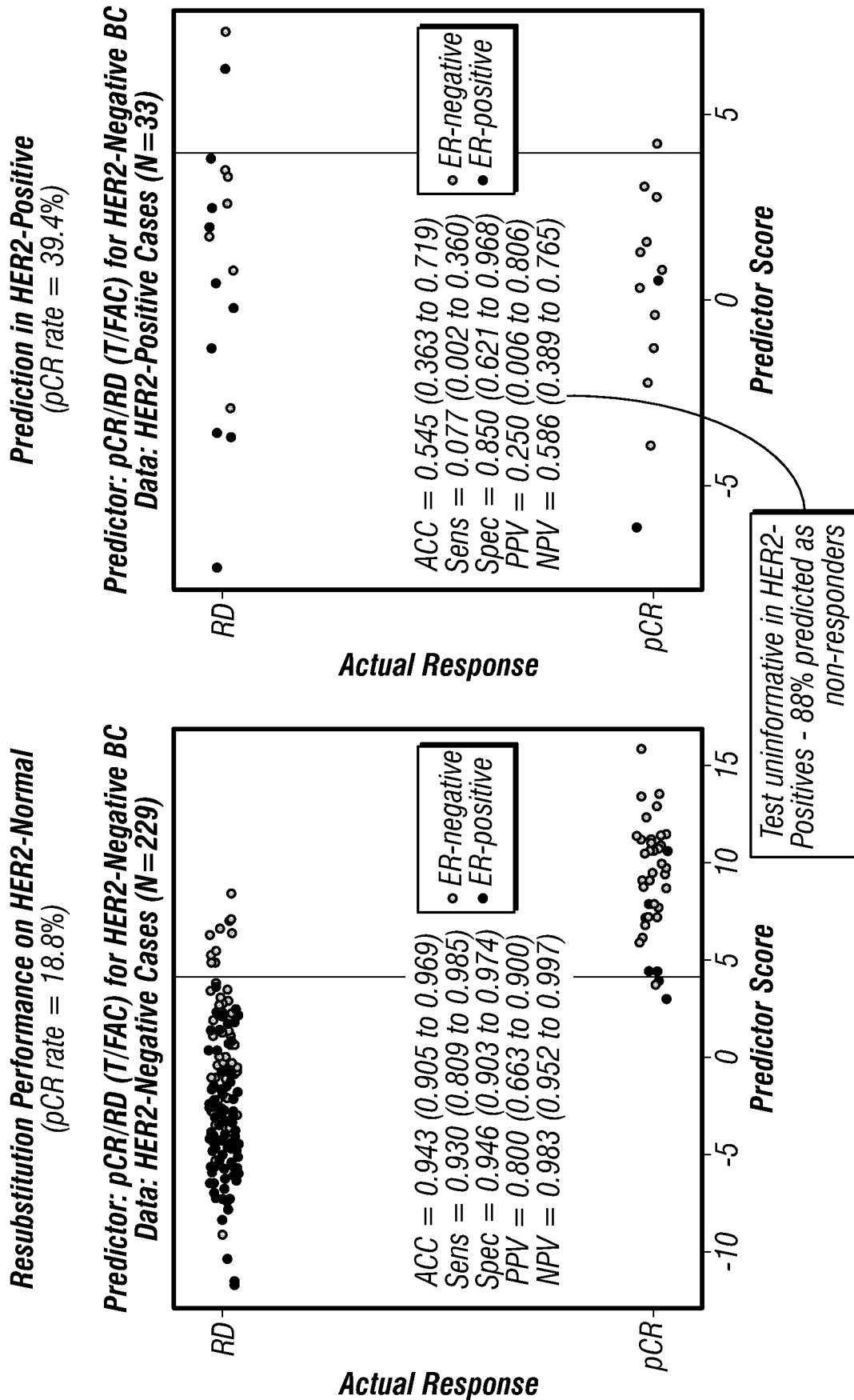


FIG. 3

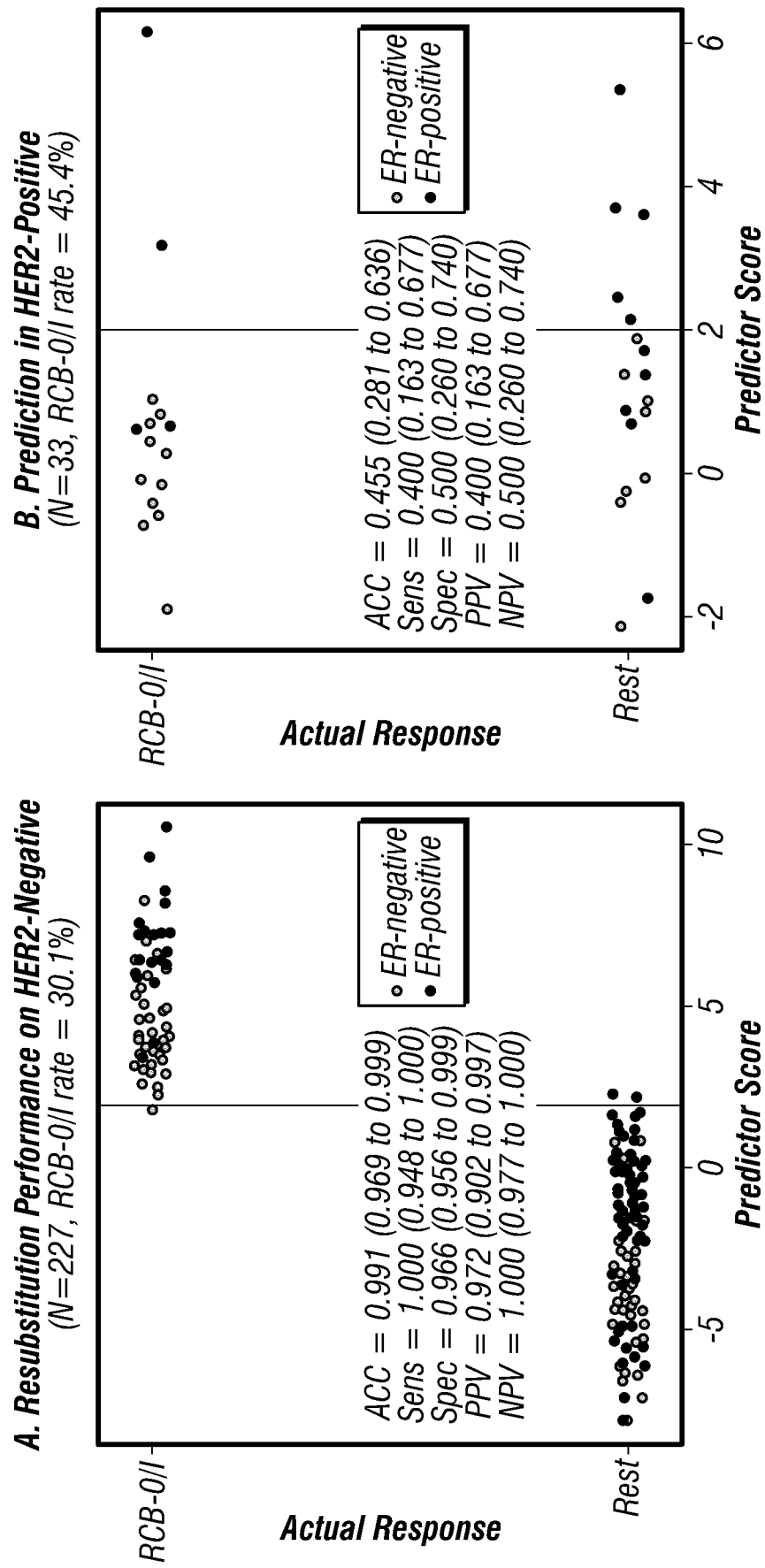
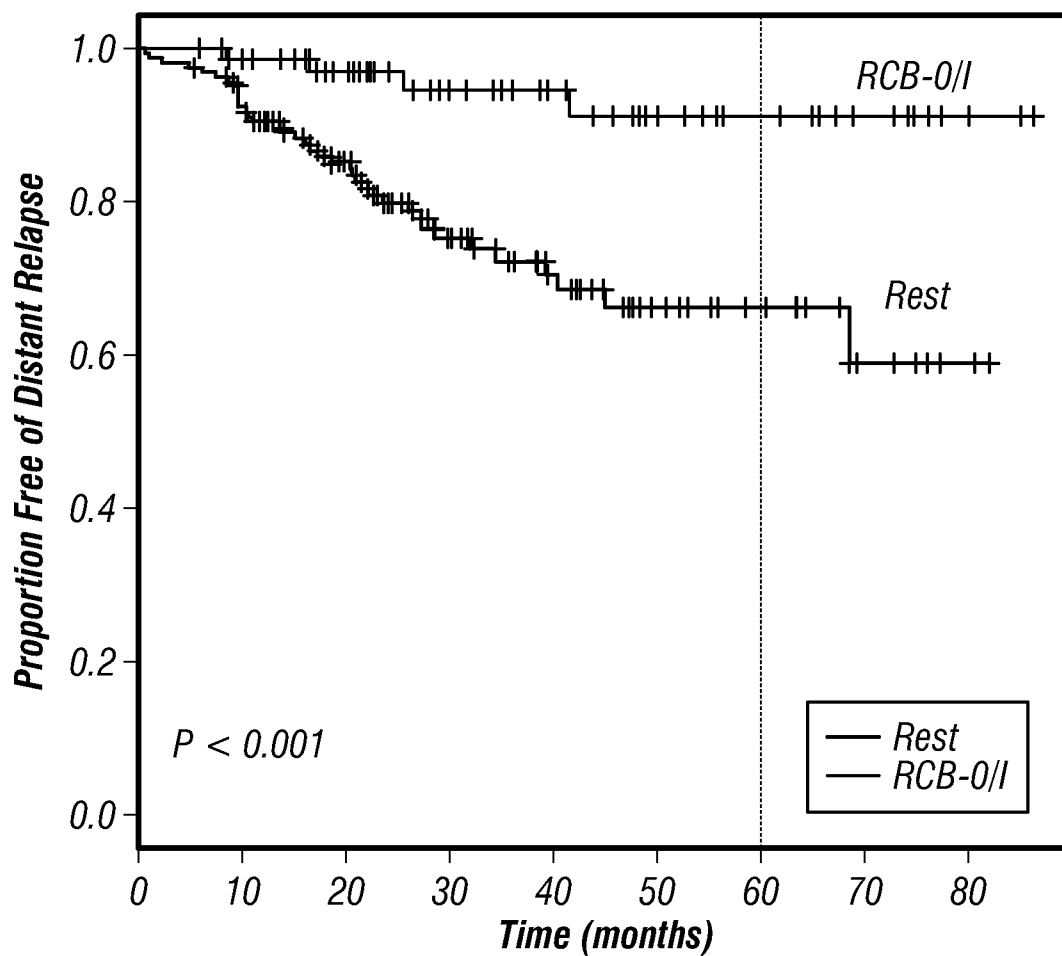


FIG. 4

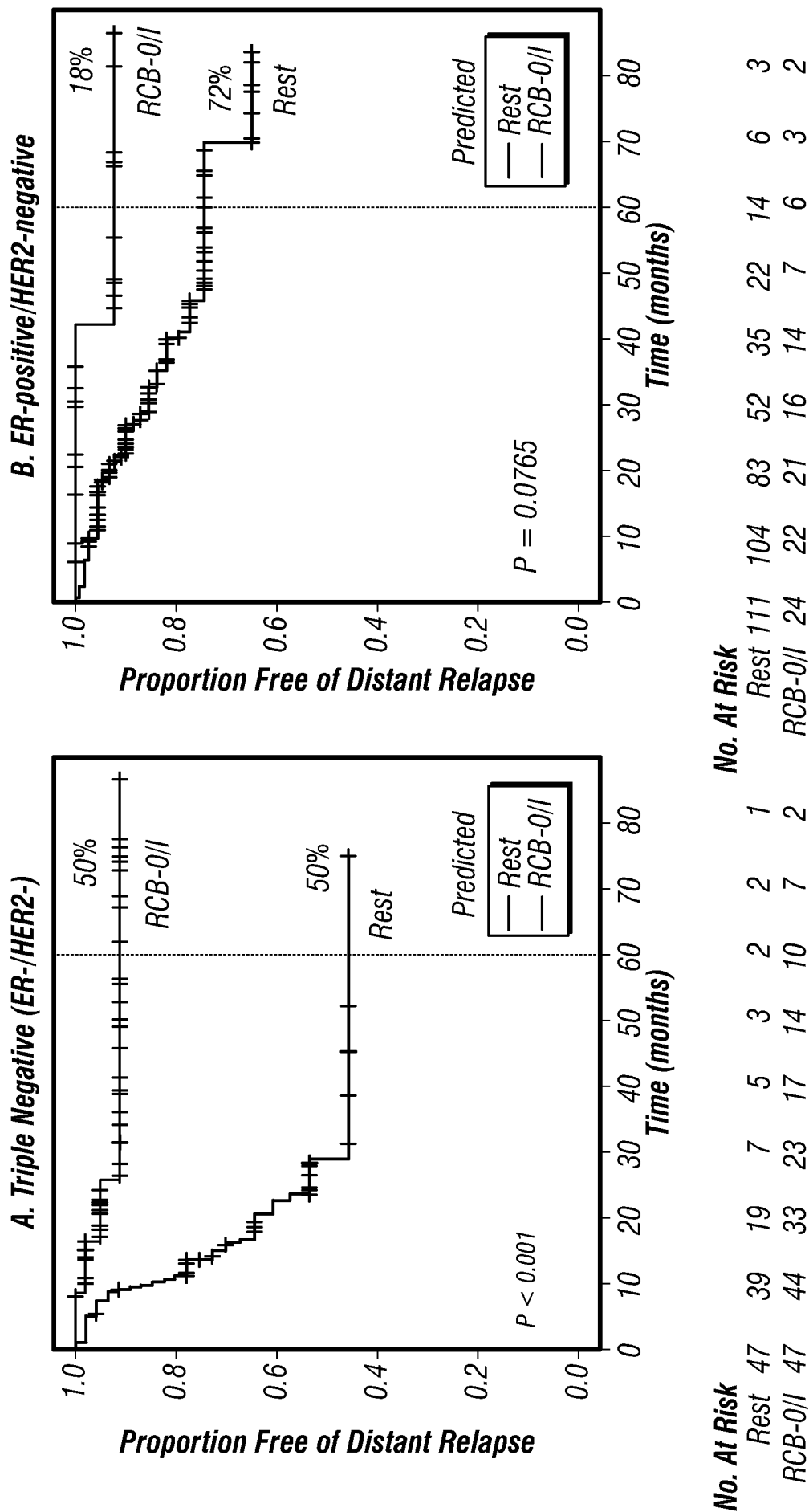
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No. At Risk

<i>Rest</i>	158	142	101	58	39	24	15	7	3
<i>RCB-0/I</i>	71	65	53	38	30	20	15	9	3

FIG. 5



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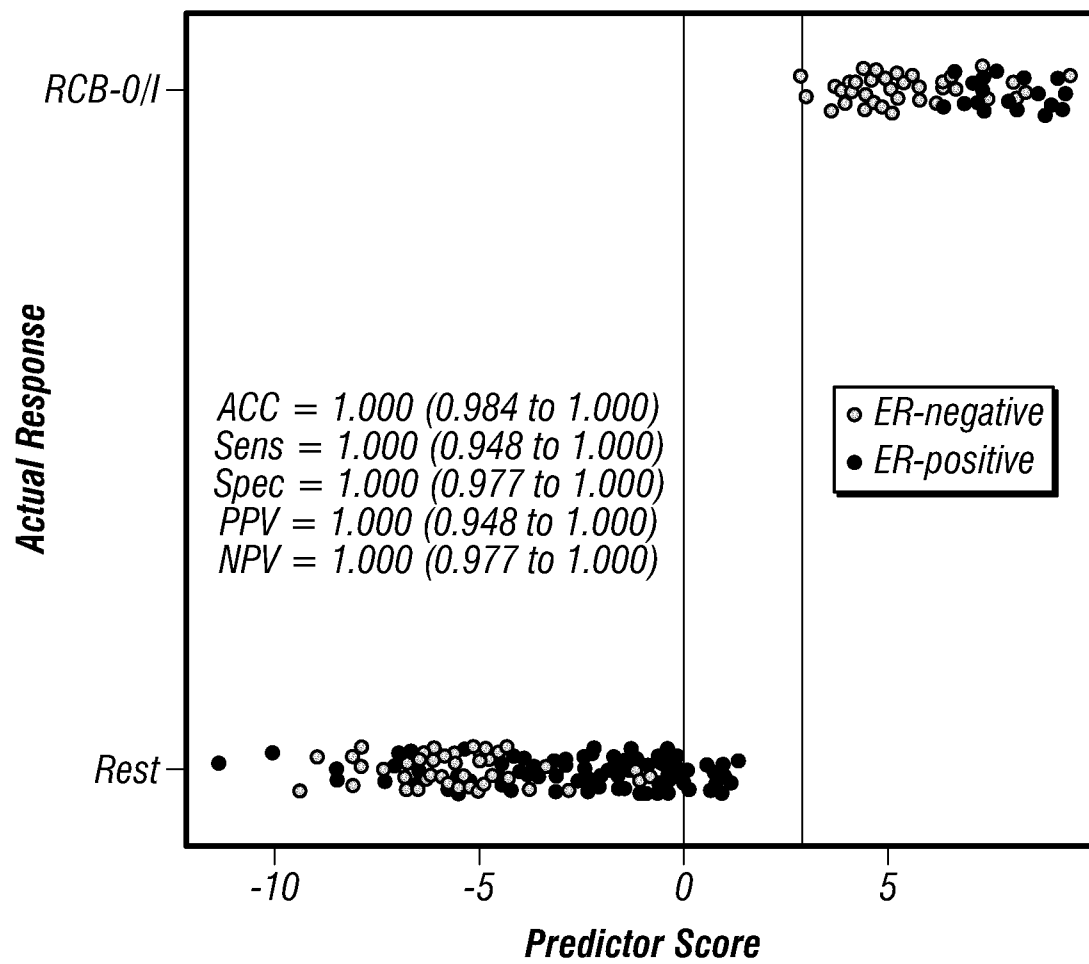
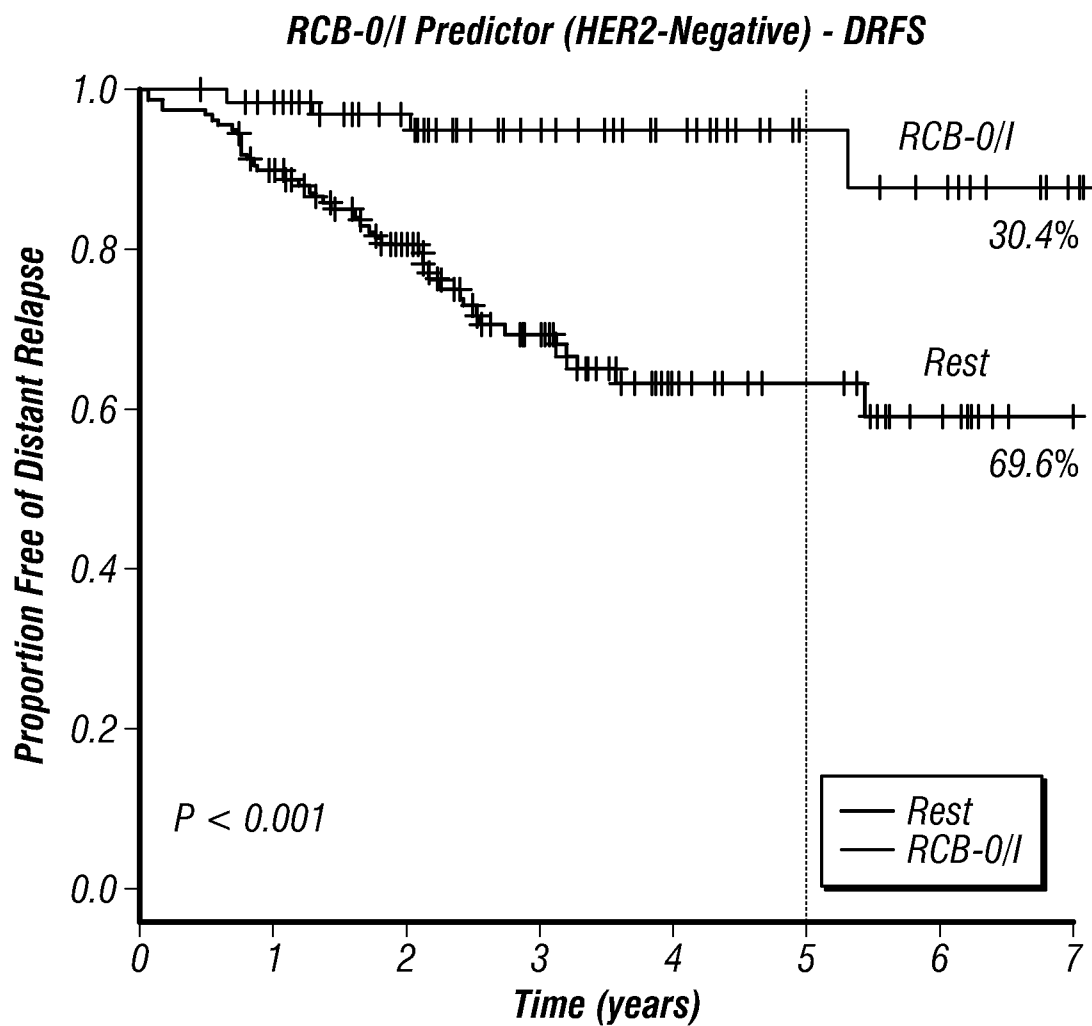


FIG. 7

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No. At Risk

Rest	158	141	98	56	32	18	11	2
RCB-0/I	69	66	49	34	27	17	12	6

FIG. 8

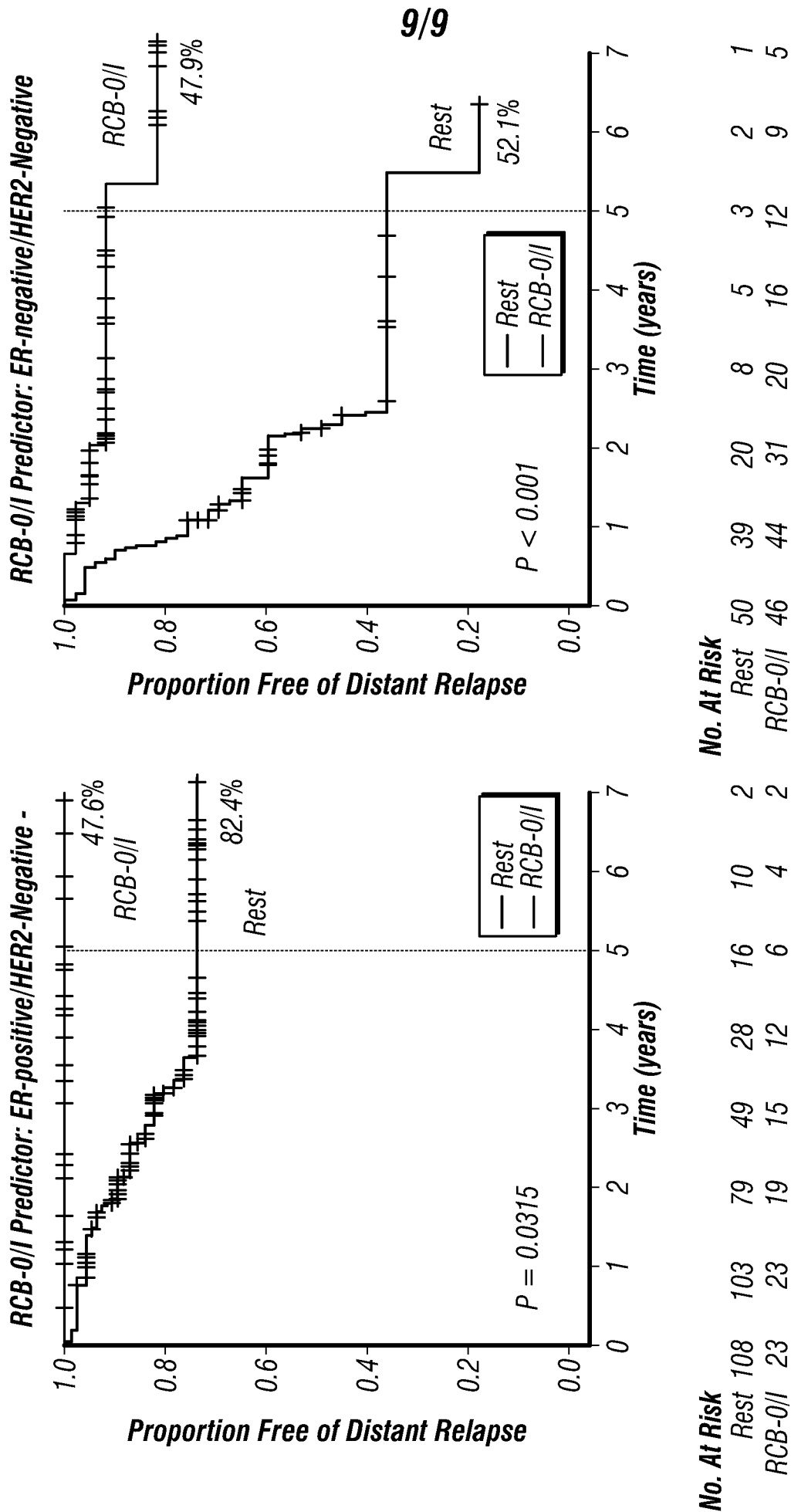


FIG. 9