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(54) Title: METHODS FOR PREDICTING RECURRENCE RISK IN BREAST CANCER PATIENTS

(57) Abstract: The invention relates to methods and kits useful for predicting or assessing recurrence risk in a patient having breast cancer who has been administered or treated with an anti-estrogen drug. Protein marker panels and methods of using the expression levels of these protein markers in calculating cancer recurrence risk and likelihood of response to the treatment are provided.



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DESCRIPTION

METHODS FOR PREDICTING RECURRENCE RISK IN BREAST CANCER PATIENTS

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[0001] This application claims priority to U.S. Application No. 61/440,213 filed on February 7, 2011, the entire disclosure of which is specifically incorporated herein by reference in its entirety without disclaimer.

TECHNICAL FIELD

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[0002] The present invention relates to protein markers, the expression levels of which correlate with likelihood of recurrence in breast cancer patients after treatment with an anti-estrogen drug.

BACKGROUND

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[0003] Cancer remains a major health concern in the United States and worldwide. For example, breast cancer is the second highest cause of cancer death in North American women (Pisani *et al.*, *Int. J. Cancer* 97:72-81, 2002; Parkin *et al.*, *Int. J. Cancer* 94:153-156, 2000). The breast cancer mortality rate in developing countries is even higher. Like many types of cancer, breast cancer is a heterogeneous disease. Clinicopathologic criteria are used to guide therapy decisions, however this approach does not define tumor biology and tumors of the same grade and stage often behave very differently. As a result, a large percentage of patients treated with chemotherapy would not have relapsed, and thus receive needless toxic therapy, while a significant proportion of patients given therapy relapse anyway. A need exists for clinical tests that help oncologists make more informed therapy decisions, a better understanding of the molecular mechanisms underlying the wide variation in cancer behavior is required.

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[0004] Using expression profiles to predict recurrence risk of breast cancer after a treatment or responsiveness to a treatment have been described. For example, the polymerase chain reaction-based Oncotype DX (Genomic Health Inc., Redwood City, CA, USA) has been used to predict response to tamoxifen. See, Paik *et al.*, *N. Engl. J. Med.* 351:2817-26, 2004; and U.S. Pat. No. 7,622,251. Although many individual proteins have been extensively studied as potential prognostic and predictive factors in breast cancer, only three are routinely accepted in current practice-estrogen receptor (ER), progesterone receptor (PR) and

HER2/neu. Generally, expression levels of ER and PR are used as a basis for selection of patients to treatment with anti-estrogen drugs, such as tamoxifen. The expression level of HER2 is used to select patients with the HER2 antagonist drugs, such as trastuzumab (Herceptin®). Often, ER positive patients are also prescribed chemotherapy in addition to anti-estrogen therapy in order to decrease the risk of cancer recurrence although a percentage of patients have low risk of cancer recurrence. Accordingly, there is a need for developing a quantitative and accurate method to identify patients who are at substantially high risk of recurrence and assist physicians to make intelligent treatment choices.

[0005] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0006] The invention provides methods for determining the likelihood of breast cancer recurrence after treatment with an anti-estrogen drug or response to a treatment with an anti-estrogen drug in an individual. The risk of recurrence and likelihood of response to the treatment is determined by calculating a recurrence risk score for said individual based on the expression levels of a panel of markers in a sample containing breast cancer cells from the individual. The panels of markers that are useful in the risk score calculation include: a two-protein panel consisting of CCNB1 and PAI1, a three-protein panel consisting of CCNB1, PR and BCL2, a four-protein panel consisting of CCNB1, PR, BCL2, and PAI1, and a five-protein panel consisting of CCNB1, PR, BCL2, ER, and GATA3. As shown in the below examples, 150 protein markers were analyzed as predictive markers for breast cancer recurrence, and none of the other protein markers or combinations were found to be as useful as these particular combinations. Thus, it was unexpected that these particular combinations of protein markers would be the most useful for predicting breast cancer recurrence. Without wishing to be bound by any theory, the evaluation of protein expression may provide substantial advantages over diagnostic tests which only evaluate mRNA levels. For example, protein expression measurement may provide significant advantages over mRNA expression levels for evaluating cancers since mRNA levels and protein levels, such as phospho-protein levels, are frequently not correlated in many cancers.

[0007] The invention provides a method for determining the likelihood of breast cancer recurrence after treatment or response to a treatment with an anti-estrogen drug in an individual, said method comprising: (a) measuring the protein expression levels of a panel of markers in a biological sample containing breast cancer cells obtained from the individual,

wherein the panel of markers comprises or is selected from the group consisting of a two-protein panel consisting of CCNB1 and PAI1, a three-protein panel consisting of CCNB1, PR and BCL2, a four-protein panel consisting of CCNB1, PR, BCL2, and PAI1, and a five-protein panel consisting of CCNB1, PR, BCL2, ER, and GATA3; (b) calculating the recurrence risk score (RS) for said individual based on the expression levels of the markers in the panel; and (c) using the RS to determine the likelihood of breast cancer recurrence or response to the treatment.

[0008] In some embodiments, the RS is the sum of the measured expression level of each marker in the panel multiplied by a coefficient reflecting the relative contribution of the expression level of the marker to cancer recurrence or response to the treatment. In some embodiments, the RS value higher than a predetermined threshold indicates an increased likelihood of breast cancer recurrence; or the RS value lower than a predetermined threshold indicates a decreased likelihood of breast cancer recurrence. Risk Score (RS) for each patient who is then classified into the lowest risk (RS smaller than or equal to the lower cutoff point), the middle risk (RS higher than the lower cutoff point but less than the upper cutoff point), or the highest risk (RS higher than or equal to the upper cutoff point) group based on the cutoff points. As shown in the below Example, Kaplan Meier survival analysis indicated that the three risk groups of the validation set were significantly different in RFS (log rank test $p < 0.01$) and that the patients in the lowest risk group had better RFS. The recurrence rate of the highest risk group was observed to be significantly higher than that of the lowest risk group ($p < 10^{-6}$).

[0009] In certain embodiments, one or more of the following statistical models may be used:

Model 1: $0.466 \times \text{"CCNB1"} - 0.183 \times \text{"PR"} - 0.199 \times \text{"BCL2"}$

Cutoff points for model 1: lower: -0.7299977; upper: -0.1393642

Model 2: $0.367 \times \text{"CCNB1"} + 0.199 \times \text{"PAI1"}$

Cutoff points for model 2: lower: -0.4238341847; upper: 0.0005819119

Model 3: $0.442 \times \text{"CCNB1"} - 0.161 \times \text{"PR"} - 0.240 \times \text{"BCL2"} + 0.213 \times \text{"PAI1"}$

Cutoff points for model 3: lower: -0.7997882; upper: -0.1449444

Model 4: $0.4175 \times \text{"CCNB1"} + 0.0898 \times \text{"ER"} - 0.191 \times \text{"PR"} - 0.2281 \times \text{"BCL2"} - 0.0733 \times \text{"GATA3"}$

Cutoff points for model 4: lower: -0.60177969; upper: -0.06292317

[0010] Each statistical model may be a multivariate Cox Proportional Hazard (COXPH) model (see, e.g., Cox, *J. R. Statistical Soc.*, B 34:187-220, 1972.), and each component in the model may be a protein marker selected by a univariate COXPH supplemented with Monte Carlo (MC) re-sampling procedure (Boos and Zhang, *J. Am. Statist. Ass.*, 95, 486-492, 2000.). For each model there are a lower and an upper cutoff points. A patient whose risk score (RS) is smaller than the lower cutoff point value has a lower risk of recurrence, between lower and upper cutoff points has an intermediate risk of recurrence, and larger than upper cutoff point has a high risk of recurrence.

[0011] In some embodiments, the coefficient for each protein is determined based on the protein expression levels in clinical samples from individuals whose risk of breast cancer recurrence or responses to the treatment are known. In some embodiments, the coefficient of CCNB1 has a positive value; the coefficient of PR has a negative value; the coefficient of BCL2 has a negative value; the coefficient of PAI1 has a positive value; the coefficient of ER has a positive value; and/or the coefficient of GATA3 has a negative value.

[0012] In some embodiments, the individual is a human. In some embodiments, said biological sample is a tumor tissue sample obtained from said human individual. In some embodiments, said biological sample is a fixed, paraffin-embedded tissue section or a fresh frozen tissue section. In embodiments, the tissue section is attached to the surface of a slide.

[0013] In some embodiments, the individual has hormone dependent breast cancer. In some embodiments, the individual has ER-positive breast cancer. In some embodiments, the individual has lymph node-negative ER-positive breast cancer. In some embodiments, the individual has lymph node-positive ER-positive breast cancer.

[0014] In some embodiments, the method is used to predict likelihood of relapse of breast cancer after tamoxifen treatment (such as 5-year relapse). In some embodiments, the breast cancer is hormone dependent. In some embodiments, the breast cancer is ER-positive. In some embodiments, the breast cancer is lymph node-negative and ER-positive. In some embodiments, the breast cancer is lymph node-positive and ER-positive.

[0015] In some embodiments, the anti-estrogen drug is an antagonist that inhibits the binding of estrogen to the estrogen receptor (such as an aromatase inhibitor and a steroid sulfatase inhibitor). In some embodiments, the anti-estrogen drug is selected from the group consisting of tamoxifen, toremifene, anastrozole, and megesterol acetate.

[0016] In some embodiments, the method further comprises a step of generating a report summarizing the result of said determination. In some embodiments, the report further

comprises information indicating whether said individual should receive treatment with said anti-estrogen drug alone, any other therapy alone, or said anti-estrogen drug plus any other therapy. In some embodiments, the other therapy is a chemotherapy.

[0017] In some embodiments, the protein expression levels are measured using reverse phase protein array. In some embodiments, the protein expression levels are measured by an immunoassay method, for example, the immunoassay method selected from the group consisting of an immunohistochemistry assay, a chemiluminescent labeled sandwich assay, and an enzyme-linked immunosorbent assay (ELISA). In various embodiments, an automated quantitative analysis (AQUA) system assay, immunoprecipitation, radioimmunoassay (RIA), immunostaining, latex agglutination, indirect hemagglutination assay (IHA), complement fixation, indirect immunofluorescent assay (FA), nephelometry, flow cytometry assay, chemiluminescence assay, lateral flow immunoassay, u-capture assay, mass spectrometry assay, particle-based assay, inhibition assay or avidity assay may be used to measure the protein expression levels.

[0018] The invention also provides a kit for determining the likelihood of breast cancer recurrence or response to a treatment with an anti-estrogen drug in an individual, comprising a panel of antibodies selected from the group consisting of 1) an antibody that specifically binds to CCNB1, and an antibody that specifically binds to PAI1; 2) an antibody that specifically binds to CCNB1, an antibody that specifically binds to PR, and an antibody that specifically binds to BCL2; 3) an antibody that specifically binds to CCNB1, an antibody that specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to PAI1; and 4) an antibody that specifically binds to CCNB1, an antibody that specifically binds to ER, an antibody that specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to GATA3.

[0019] In some embodiments, the antibody in the panel is conjugated to a label. In some embodiments, the label is a fluorophore or an enzyme. In some embodiments, each antibody in a panel is conjugated to a different label. In some embodiments, the antibodies in the kit specifically bind to human marker proteins.

[0020] In some embodiments, the kit further comprises a package insert providing instructions for measuring the protein expression levels of the markers in a panel in a biological sample from the individual and/or determining the recurrence risk and likelihood of response to the treatment based on the expression levels of the markers in the panel.

[0021] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present

invention. These and other aspects of the invention will become apparent to one of skill in the art

BRIEF DESCRIPTION OF THE FIGURES

[0022] FIGS. 1A-D: shows the Receiver Operating Characteristic (ROC) curves of the 2-protein model (CCNB1 and PAI1) (FIG. 1A), 3-protein model (CCNB1, PR and BCL2) (FIG. 1B), 4-protein model (CCNB1, PR, BCL2, and PAI1) (FIG. 1C), and 5-protein model (CCNB1, ER, PR, BCL2, AND GATA3) (FIG. 1D). The ROC curves plot true positives (sensitivity) versus false positives (1-specificity). The area under curve (AUC) indicates the model's performance in prediction of 5-year recurrence.

[0023] FIGS. 2A-D shows the Kaplan Meier survival analysis of data from 77 estrogen receptor positive breast cancer patients treated with tamoxifen. Lymph node status for these patients was not available. Using the algorithm and cutoff points disclosed in Example 1 for the 2-protein model (CCNB1 and PAI1), 3-protein model (CCNB1, PR and BCL2), 4-protein model (CCNB1, PR, BCL2, and PAI1), and 5-protein model (CCNB1, ER, PR, BCL2, AND GATA3), respectively, patients were divided into high-risk, middle-risk, and low-risk groups. The proportions of recurrence-free survival (RFS) patients in the high-risk, middle-risk, and low-risk groups over time (months) are shown in the graphs (FIG. 2A: 2-protein model; FIG. 2B: 3-protein model; FIG. 2C: 4-protein model; and FIG. 2D: 5-protein model). Data indicates that the low-risk patient group has the highest proportion of RFS.

[0024] FIG. 3 presents results of Kaplan-Meier survival analysis from a clinical study of 123 tamoxifen treated patients with lymph node-negative, estrogen receptor positive breast cancer. Recurrence data for 91 patients were available. Using the algorithm and cutoff points disclosed in Example 1 for the 3-protein model (CCNB1, PR and BCL2), patients were divided into high-risk, middle-risk, and low-risk groups. The proportions of recurrence-free survival (RFS) patients in the high-risk, mid-risk, and low-risk groups over time are shown in the graph. Data indicates that the low-risk patient group has the highest proportion of RFS.

[0025] FIGS. 4A-D: Kaplan-Meier overall survival curves are shown for the 2-protein model (CCNB1 and PAI1; FIG. 4A), 3-protein model (CCNB1, PR and BCL2; FIG. 4B), 4-protein model (CCNB1, PR, BCL2, and PAI; FIG. 4C), and 5-protein model (CCNB1, ER, PR, BCL2, and GATA3; FIG. 4D), respectively, using an independent validation set, TCGA breast cancer set.

DETAILED DESCRIPTION

[0026] The invention provides methods and kits for predicting recurrence risk in breast cancer patients after treatment with an anti-estrogen drug, and for predicting response to a treatment with an anti-estrogen drug. The methods described herein comprise a step of measuring the protein expression levels of one or more of marker panels in a breast cancer tissue sample from an individual. In some embodiments, the protein marker panel comprises CCNB1 (cyclin B1) and PAI1 (plasminogen activator inhibitor type-1). In some embodiments, the protein marker panel comprises CCNB1, PR (progesterone receptor), and BCL2. In some embodiments, the protein marker panel comprises CCNB1, PR, BCL2, and PAI1. In some embodiments, the protein marker panel comprises CCNB1, ER (estrogen receptor), PR, BCL2, and GATA3. The methods may further comprise a step of calculating the recurrence risk score (RS) for the individual using a model generated based on the protein expression levels of a panel of markers in clinical samples from individuals whose risk of breast cancer recurrence after treatment or response to the treatment are known. For example, the RS can be calculated as the sum of the measured expression level of each marker in a panel multiplied by a coefficient reflecting the relative contribution of the expression level of the protein to cancer recurrence after treatment or response to the treatment. The method may further comprise a step of using the RS to predict breast cancer recurrence after treatment or response to the treatment.

A. Definitions

[0027] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

[0028] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al., Dictionary of Microbiology and Molecular Biology 2nd

ed., J. Wiley & Sons (New York, N.Y. 1994), and March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, N.Y. 1992), provide one skilled in the art with a general guide to many of the terms used in the present application

5 **[0029]** As used herein, the term “sample” or “biological sample” refers to a composition that is obtained or derived from an individual of interest that contains a cellular and/or other molecular entity that is to be characterized and/or identified, for example based on physical, biochemical, chemical and/or physiological characteristics. Samples include tissue and cell sample from a tissue of an individual. The cell sample may comprise
10 cancerous cells or cells from a tumor, such as a cancerous tumor. In certain embodiments, the sample comprises breast cancer cells. The source of the tissue or cell sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of
15 the subject. The tissue sample may also be primary or cultured cells or cell lines. Optionally, the tissue or cell sample is obtained from a disease tissue/organ. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like

[0030] As used herein, a “section” of a tissue sample is meant a single part or piece
20 of a tissue sample, *e.g.* a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis according to the present invention, provided that it is understood that the present invention comprises a method whereby the same section of tissue sample is analyzed at both morphological and molecular levels, or is analyzed with respect to protein or nucleic acid.

25 **[0031]** The term “tumor,” as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

[0032] The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples
30 of cancer include, but are not limited to, breast cancer, ovarian cancer, colon cancer, lung cancer, prostate cancer, hepatocellular cancer, gastric cancer, pancreatic cancer, cervical cancer, liver cancer, bladder cancer, cancer of the urinary tract, thyroid cancer, renal cancer, carcinoma, melanoma, and brain cancer.

[0033] The term "long-term" survival is used herein to refer to survival for at least 3 years, at least 5 years, at least 8 years, or at least 10 years following therapeutic treatment

[0034] A "subject" or an "individual" is a mammal, more preferably a human. Mammals include, but are not limited to, humans, primates, farm animal, sport animals, rodents, and pets (*e.g.*, dogs and cats).

[0035] As used herein, the term "treatment" refers to clinical intervention designed to alter the natural course of the individual or cell being treated during the course of clinical pathology. Desirable effects of treatment include decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis. An individual is successfully "treated", for example, if one or more symptoms associated with the disease or condition are mitigated or eliminated. For example, significant reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (*i.e.*, slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; increase in length of remission, and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life.

[0036] The term "prediction" is used herein to refer to the likelihood that a patient will respond either favorably or unfavorably to a drug or set of drugs. In one embodiment, the prediction relates to the extent of those responses. In one embodiment, the prediction relates to the risk of cancer recurrence after a therapy (such as treatment with an estrogen receptor antagonist for a certain period of time). In one embodiment, the prediction relates to whether and/or the probability that a patient will survive, following a therapy (such as treatment with an estrogen receptor antagonist for a certain period of time) without cancer recurrence. The predictive methods of the present invention can be used clinically to make treatment decisions by choosing the most appropriate treatment modalities for any particular patient. The predictive methods of the present invention may be used to predict, *e.g.*, if a patient is likely to respond favorably to a treatment regimen, or whether long-term survival of the patient following a treatment regimen is likely.

[0037] As used herein, method for "aiding assessment" refers to methods that assist in making a clinical determination (*e.g.*, responsiveness of a breast cancer patient to treatment with an antagonist of estrogen receptor), and may or may not be conclusive with respect to the definitive assessment.

[0038] "Patient response" can be assessed using any endpoint indicating a benefit to the patient, including, without limitation, (1) inhibition, to some extent, of disease

progression, including slowing down and complete arrest; (2) reduction in the number of disease episodes and/or symptoms; (3) reduction in lesional size; (4) inhibition (*i.e.*, reduction, slowing down or complete stopping) of disease cell infiltration into adjacent peripheral organs and/or tissues; (5) inhibition (*i.e.*, reduction, slowing down or complete stopping) of disease spread; (6) relief, to some extent, of one or more symptoms associated with the disorder; (7) increase in the length of disease-free presentation following treatment; and/or (8) decreased mortality at a given point of time following treatment.

[0039] The term "node negative" cancer, such as "node negative" breast cancer, is used herein to refer to cancer that has not spread to the lymph nodes.

[0040] The term "antibody" is used in the broadest sense and specifically covers polyclonal antibodies, monoclonal antibodies (including full length monoclonal antibodies), and antibody fragments so long as they exhibit the desired biological activity or function.

[0041] The term "specifically recognizes" or "specifically binds" refers to measurable and reproducible interactions such as a selective attraction or binding between a target and an antibody, that is determinative of the presence and/or the amount of the target in the presence of a heterogeneous population of molecules including biological molecules. An antibody that specifically binds to a target may have an association constant of at least about 10^3 M^{-1} to 10^4 M^{-1} , sometimes about 10^5 M^{-1} to 10^6 M^{-1} , in other instances about 10^6 M^{-1} to 10^7 M^{-1} , about 10^8 M^{-1} to 10^9 M^{-1} , or about 10^{10} M^{-1} to 10^{11} M^{-1} or higher. A variety of immunoassay formats can be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See, *e.g.*, Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

[0042] The term "array" or "microarray", as used herein refers to an ordered arrangement of hybridizable or binding array elements, such as polynucleotide probes (*e.g.*, oligonucleotides) and antibodies, on a substrate. The substrate can be a solid substrate, such as a glass slide, or a semi-solid substrate, such as nitrocellulose membrane. The antibodies can be monoclonal antibodies or fragments thereof.

[0043] The term "biomarker" or "marker" as used herein refers generally to a molecule, including a gene, protein, carbohydrate structure, or glycolipid, the expression of which in or on a mammalian tissue or cell or secreted can be detected by known methods (or methods disclosed herein) and is predictive or can be used to predict (or aid prediction) for a

mammalian cell's or tissue's sensitivity to, and in some embodiments, to predict (or aid prediction) an individual's responsiveness to treatment regimes.

[0044] As used herein, the term "label" refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

[0045] As used herein, "a", "an", and "the" can mean singular or plural (*i.e.*, can mean one or more) unless indicated otherwise. 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."

[0046] It is understood that aspect and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

B. Methods of the Invention

[0047] The invention provides methods for predicting recurrence risk in breast cancer patients after treatment with an anti-estrogen drug and response to a treatment with an anti-estrogen drug. The invention may include steps of measuring protein expression levels of a panel of markers and using the expression levels for determining the recurrence risk and response to a treatment.

Measuring expression levels of marker proteins

[0048] The methods disclosed herein provide methods for measuring the expression levels of one or more of the protein markers in a tissue or a cell sample from an individual having breast cancer. Methods known in the art may be used for measuring the expression levels.

[0049] The sample can be obtained by a variety of procedures known in the art including, but is not limited to surgical excision, aspiration or biopsy. For example, for obtaining patient samples, H&E staining may be carried out and used as a guide for tissue macrodissection to enrich for tumor content. The sample may be fresh or frozen. In some embodiments, the sample is fixed and embedded in paraffin or the like. The samples

obtained may be further treated (for example lysed) for use in the methodologies for detecting the protein marker expression levels.

[0050] Expression of various protein markers in a sample can be analyzed by a number of methodologies, many of which are known in the art and understood by the skilled artisan including, but not limited to, immunohistochemical and/or Western analysis, FACS, protein arrays, mass spectrometry, quantitative blood based assays (e.g., serum ELISA), an enzyme-linked immunoassay, an AQUA system assay, a radioimmunoassay, an immunoprecipitation, a fluorescence immunoassay, a chemiluminescent assay, an immunoblot assay, a lateral flow assay, a flow cytometry assay, a Bio-Plex suspension array assay, a dipstick test, and a particulate-based assay (e.g., a particulate-based suspension array assay performed using the Bio-Plex® system; Bio-Rad Laboratories, Hercules, CA, USA).

Reverse Phase Protein Array (RPPA)

[0051] In some embodiments, reverse phase protein array described in U.S. Publication No. 2008/0108091 is used for measuring the expression levels of the marker proteins. Tissue or cellular lysates can be obtained by mixing tissue sample material with lysis buffer and then serially diluted (e.g., 8 serial dilutions: full strength, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) with additional lysis buffer. In some embodiments, 4, 5, or 6 serial dilutions are used. Dilutions can be automated, for example, using a Tecan liquid handling robot or other similar device. This material can be printed/spotted onto a substrate, such as nitrocellulose-coated glass slides (FAST Slides, Schleicher & Schuell BioScience, Inc. USA, Keene, N.H.) with an automated GeneTac arrayer (Genomic Solutions, Inc., Ann Arbor, Mich.) or other similar devices. In certain embodiments, as many as 1,056 samples can be spotted in 5 serial dilutions on a single substrate. Serial dilutions can provide a slope and intercept allowing relative quantification of individual proteins. Typically, measurements of protein are compared to control peptides allowing absolute quantification.

[0052] Typically, after slide printing, the same stringent conditions for slide blocking, blotting and antibody incubation used for Western blotting may be applied prior to the addition of the primary antibody. The DAKO (Copenhagen, Denmark) signal amplification system can be used to detect and amplify antibody-binding intensity. Signal intensity is measured by scanning the slides and quantifying with software, such as the MicroVigene automated RPPA software (VigeneTech Inc., Massachusetts), to generate sigmoidal signal intensity-concentration curves for each sample. To accurately determine absolute protein concentrations, standard signal intensity-concentration curves for purified

proteins/recombinant peptides of known concentration are generated for comparison with the samples in which protein concentrations are unknown. The RPPAs are quantitative, sensitive, and reproducible. RPPA may also be validated with mTOR, erk, p38, GSK3 and JNK as stable loading controls.

5

Immunohistochemistry

[0053] Immunohistochemistry (IHC) methods are also suitable for detecting or measuring the expression levels of the protein markers of the present invention. Thus, antibodies or antisera (including polyclonal antisera and monoclonal antibodies) specific for each marker are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

[0054] Tissue samples from an individual having breast cancer may be fixed and processed prior to IHC staining. Fixation prevents autolysis and necrosis of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing may include dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues.

[0055] A tissue sample may be fixed (*i.e.* preserved) by conventional methodology (See *e.g.*, “Manual of Histological Staining Method of the Armed Forces Institute of Pathology,” 3rd edition (1960)). The choice of a fixative may be determined by the purpose for which the sample is to be histologically stained or otherwise analyzed. The length of fixation depends upon the size of the tissue sample and the fixative used. By way of example, neutral buffered formalin, Bouin’s or paraformaldehyde, may be used to fix a sample.

[0056] After fixation, the sample may be dehydrated through an ascending series of alcohols, infiltrated and embedded with paraffin or other sectioning media so that the tissue sample may be sectioned. Alternatively, one may section the tissue and fix the sections obtained. Once the tissue sample is embedded, the sample may be sectioned by a microtome or the like (See *e.g.*, “Manual of Histological Staining Method of the Armed Forces Institute

of Pathology”, *supra*). Sections may range from about three microns to about five microns in thickness. Once sectioned, the sections may be attached to slides by several standard methods. Examples of slide adhesives include, but are not limited to, silane, gelatin, poly-L-lysine and the like. In some embodiments, the paraffin embedded sections may be attached to positively charged slides and/or slides coated with poly-L-lysine.

[0057] If paraffin is used as the embedding material, the tissue section may be deparaffinized to remove embedding media and rehydrated. Methods to deparaffinize the tissue sections are known in the art. For example, xylenes and a gradually descending series of alcohols may be used (*See e.g.*, “Manual of Histological Staining Method of the Armed Forces Institute of Pathology”, *supra*). Alternatively, commercially available deparaffinizing non-organic agents such as Hemo-De7 (CMS, Houston, Texas) may be used.

[0058] Subsequent to the sample preparation, a tissue section may be analyzed using IHC. Further treatment of the tissue section prior to, during or following IHC may be desired. For example, proteolytic digestion and/or protein target retrieval methods, such as heating the tissue sample in citrate buffer may be carried out before prior to IHC staining (*see, e.g.*, Leong *et al. Appl. Immunohistochem.* 4(3):201 (1996)).

[0059] A tissue section may be blocked before the antibody staining step. For antibody staining, a tissue section may be exposed to a primary antibody for a sufficient period of time and under suitable conditions such that the primary antibody binds to the target protein antigen in the tissue sample. Appropriate conditions for achieving this can be determined by routine experimentation. The extent of binding of an antibody to the sample is determined by using any one of the detectable labels.

[0060] The primary antibody may be labeled, and binding of the primary antibody to the protein marker is determined directly. In some embodiments, primary antibodies for different markers may be conjugated to different labels, and all markers may be detected on a single tissue section. Alternatively, a labeled secondary antibody that binds to the primary antibody may be used for detecting and measuring the binding of the primary antibody to the tissue section.

[0061] Specimens thus prepared may be mounted and coverslipped. Slide evaluation is then determined, *e.g.* using a microscope, staining intensity criteria, and digital imaging systems (such as digital imaging systems from CRi, Aperio, Bioimaging, and HistoRx) routinely used in the art, may be employed.

Antibodies that specifically bind to the protein markers

[0062] The invention also provides antibodies that specifically bind to or recognize the protein markers disclosed herein. Antibodies can be made by any of the methods that as well known to those of skill in the art. The following methods exemplify some of the most common antibody production methods.

[0063] Polyclonal antibodies generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen. As used herein the term "antigen" refers to any polypeptide that comprises a portion of or the full length protein of the protein markers described herein. However, it will be understood by one of skill in the art that in many cases antigens comprise more material than merely a single polypeptide. In certain other aspects of the invention, antibodies will be generated against specific polypeptide antigens. In some cases the full length polypeptide sequences may be used as an antigen however in certain cases fragments of a polypeptide (i.e., peptides) may be used. In still further cases, antigens may be defined as comprising or as not comprising certain post translational modifications such, phosphorylated, acetylated, methylated, glycosylated, prenylated, ubiquitinated, sumoylated or NEDDylated residues. In another example, antibodies can be made against polypeptides that have been identified to be expressed on the surface of cancer cells, such as ER. Thus one skilled in the art would easily be able to generate an antibody that binds to any particular cell or polypeptide of interest using methods that are well known in the art.

[0064] In the case where an antibody is to be generated that binds to a particular polypeptide it may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g. keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glytaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1 \text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

[0065] Animals are immunized against the immunogenic conjugates or derivatives by, for example, combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freud's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with about 1/5 to 1/10 the original amount of conjugate in Freud's complete adjuvant by subcutaneous injection at multiple sites. Seven to

14 days later the animals are bled and the serum is assayed for specific antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the same antigen conjugate, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents, such as alum, or other adjuvants may be used to enhance the immune response.

[0066] The invention also provides monoclonal antibodies for detecting and measuring the expression levels of the protein markers described herein. Monoclonal antibodies may be obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Monoclonal antibodies include, but are not limited to, mouse monoclonal antibodies, rabbit monoclonal antibodies, human monoclonal antibodies, and chimeric antibodies.

[0067] For example, monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein (*Nature* 256:495-497, 1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

[0068] In the hybridoma method, a mouse or other appropriate host animal (such as a rabbit) is immunized as described above to elicit lymphocytes, such as plasma cells, that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes may then be fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, Orlando, Fla., pp60-61, 71-74, 1986).

[0069] The hybridoma cells thus prepared may be seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0070] Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine

myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Md. USA.

5 [0071] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the target antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson & Pollard
10 (1980).

[0072] After hybridoma cells are identified that produce antibodies of the desired specificity (e.g., specificity for a phosphorylated vs. un-phosphorylated antigen), affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, 2d ed.,
15 Academic Press, Orlando, Fla., pp60-61, 71-74, 1986). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

[0073] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin
20 purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0074] Rabbit monoclonal antibodies may also be used for measuring expression levels of the marker proteins. Methods for generating rabbit monoclonal antibodies are known in the art. See U.S. Pat. Nos. 5,675,063 and 7,429,487, and Spieker-Polet et al., *Proc.*
25 *Natl. Acad. Sci. USA* 92:9348-9352, 1995.

[0075] DNA encoding the monoclonal antibodies of the invention may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such
30 DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant

domains in place of the homologous murine sequences (Morrison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-6833, 1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity for any particular antigen described herein.

[0076] Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for the target antigen and another antigen-combining site having specificity for a different antigen. Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry. Other methods known in the art, such as phage display and yeast display, may also be used to generate antibodies that specific bind to the protein markers.

[0077] For some applications, the antibodies of the invention will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; biotin (which enables detection of the antibody with an agent that binds to biotin, such as avidin; or an enzyme (either by chemical coupling or polypeptide fusion), such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

[0078] Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter *et al.*, *Nature* 144:945, 1962; David *et al.*, *Biochemistry* 12:1014, 1974; Pain *et al.*, *J. Immunol. Meth.* 40:219, 1981; and Nygren, *J. Histochem. Cytochem.* 30:407-412, 1982.

[0079] The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays (Zola, In: *Molecular Antibodies: A Manual of Techniques*, CRC Press, Inc., pp 147-158, 1987). For instance the antibodies may be used in the detection assays described herein.

[0080] Additionally, antibodies may be used in competitive binding assays. These assays rely on the ability of a labeled standard (which may be a purified target antigen or an immunologically reactive portion thereof) to compete with the test sample analyte for binding with a limited amount of antibody. The amount of antigen in the test sample is inversely

proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

[0081] Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex (see for example U.S. Pat. No. 4,376,110). The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

[0082] Commercially available antibodies against the protein markers may be used for measuring expression levels of protein markers. For example, anti-human BCL2 antibody from Dako (Carpinteria, CA, USA), anti-human ER antibody from Lab Vision Corporation (Fremont, CA, USA), anti-human CCNB1 antibody and anti-human PR antibody from Epitomics Inc. (Burlingame, CA, USA), and anti-human GATA3 antibody and anti-human PAI1 antibody from BD Biosciences (San Jose, CA, USA) may be used.

Lateral Flow Tests

[0083] Lateral flow tests may also be referred to as immunochromatographic strip (ICS) tests or simply strip-tests. In general, a lateral flow test is a form of assay in which the test sample flows laterally along a solid substrate via capillary action, or alternatively, under fluidic control. Such tests are often inexpensive, require a very small amount (*e.g.*, one drop) of sample, and can typically be performed reproducibly with minimal training.

[0084] Exemplary lateral flow device formats include, but are not limited to, a dipstick, a card, a chip, a microslide, and a cassette, and it is widely demonstrated in the art that the choice of format is largely dependent upon the features of a particular assay. Lateral flow devices provide many options to the ordinarily skilled artisan for detecting a protein-antibody complex in a sample using a lateral flow assay (*e.g.*, U.S. Patents 7,344,893, 7,371,582, 6,136,610, and U.S. Patent Applications, 2005/0250141 and 2005/0047972, each incorporated herein by reference.)

[0085] In related embodiments, an ELISA assay may be performed in a rapid flow-through, lateral flow, or strip test format. Various methods of detection may be used in a lateral flow immunoassay including, for example, the detection of a colored particle (e.g., latex, gold, magnetic particle, fluorescent particle). In certain embodiments, a lateral flow assay may comprise a sandwich ELISA assay specific for a protein marker.

Particulate-Based Assays

[0086] In general, particle-based assays use a capture-binding partner, such as an antibody or an antigen in the case of an immunoassay, coated on the surface of particles, such as microbeads, crystals, chips, or nanoparticles. Particle-based assays may be effectively multi-plexed or modified to assay numerous variables of interest by incorporating fluorescently labeled particles or particles of different sizes in a single assay, each coated or conjugated to one or more labeled capture-binding partners. The use of sensitive detection and amplification technologies with particle-based assay platforms known in the art has resulted in numerous flexible and sensitive assay systems to choose from in performing a method described herein. For example, a multi-plex particle-based assay such as the suspension array Bio-Plex® assay system available from Bio-Rad Laboratories, Inc. (Hercules, CA) and Luminex, Inc. (Austin, TX) may be useful in evaluating expression of protein marker in a sample.

Automated Quantitative Analysis (AQUA) of Protein Expression

[0087] The automated quantitative analysis (AQUA) system may be used to detect expression of a panel of markers or a combination of biomarkers, as described herein. The AQUA system is an automated scoring system for assessing biomarker expression in tissue sections (e.g., see Camp *et al.*, *Nat Med* 2002;8:1323–7; McCabe *et al.*, *JNCI J Natl Cancer Inst* 2005, 97 (24): 1808-1815; which are incorporated by reference in their entirety). The AQUA system is typically linked to a fluorescent microscope system that detects the expression of biomarker proteins by measuring the intensity of antibody-conjugated fluorophores within a specified subcellular compartment (typically including the nucleus, cytoplasm, and plasma membrane) within the tumor region of each tissue microarray spot. The result is a quantitative score of immunofluorescence intensity for the tumor. An AQUA analysis removes the subjectivity of certain traditional scoring systems and provides a more continuous and reproducible scoring of protein expression scoring in tissue samples (Camp *et al.*, *Nat Med* 2002;8:1323–7).

[0088] A tissue microarray may be generated for use with AQUA via the following protocol. Tissue microarrays may be constructed from tumor tissue core samples. Core samples may be obtained from representative regions of sections from each tumor that has been selected by use of the corresponding full sections stained with hematoxylin and eosin.

5 The tissue microarray may be constructed with single core samples (about 0.6-mm diameter) from each tumor and spaced about 0.8 mm apart in a grid format by using a tissue microarrayer (Beecher Instruments, Silver Spring, MD) (*e.g.*, Camp *et al.*, *Lab Invest* 2000; 80:1943–9.). The tissue microarray may be cut into about 5- μ m sections with a microtome, and the sections may be adhered to the slide by means of an adhesive tape-transfer method, as
10 described by the manufacturer (Instrumedics, Inc., Hackensack, NJ), and cross-linked to the slide with UV irradiation according to manufacturer's instructions.

[0089] Tissue microarrays may be immunohistochemically stained as described previously (*e.g.*, see Camp *et al.*, *Nat Med* 2002;8:1323–7; McCabe *et al.*, *JNCI J Natl Cancer Inst* 2005, 97 (24): 1808-1815). In certain embodiments, the following method may
15 be used to immunostain the tissue microarray. The tissue microarray slides may be deparaffinized by two xylene rinses followed by two rinses with 100% ethanol. Antigen retrieval may be performed by boiling the slides in a pressure cooker filled with 7.5 mM sodium citrate (pH 6.0). After rinsing briefly in 1 $\times$ Tris-buffered saline (TBS) at pH 8, slides may be incubated for 30 minutes in 2.5% hydrogen peroxide in methanol to block
20 endogenous peroxidase activity. Slides may then be incubated with 0.3% bovine serum albumin in 1 $\times$ TBS for 1 hour at room temperature to reduce nonspecific background staining and then subjected to washes in 1 $\times$ TBS, in 1 $\times$ TBS containing 0.01% Triton, and then in 1 $\times$ TBS, each 2 minutes long (hereafter referred to as TBS rinses). Slides may be incubated first
25 with a mouse anti-cytokeratin monoclonal antibody (*e.g.*, clone AE1/AE3, DAKO, Carpinteria, CA; diluted 1:200) or with a rabbit anti-cytokeratin polyclonal antibody (*e.g.*, Zymed, South San Francisco, CA; diluted 1:50) overnight at 4 °C to define the epithelial mask. Slides may be rinsed in 1 $\times$ TBS and then incubated with an antibody specific for or
which selectively binds CCNB1, PR, BCL2, PAI1, ER, GATA3, or EIG121, *e.g.*, for about 1 hour at room temperature. Slides may be rinsed in TBS as described above and incubated
30 with secondary antibodies for 1 hour at room temperature, *e.g.*, biotin anti-mouse or biotin goat anti-rabbit secondary antibodies (*e.g.*, Vector Laboratories, Burlingame, CA; diluted 1:200) or mouse or rabbit secondary antibodies attached to a dextran-polymer backbone that was decorated with more than 100 molecules of covalently attached horseradish peroxidase. The slides may be washed with the TBS rinses described above and incubated for 30 minutes

at room temperature with Alexa 546-streptavidin (*e.g.*, Molecular Probes, Eugene, OR; diluted 1: 200) to label the cytokeratin and then for 10 minutes with Cy-5 tyramide (NEN Life Science Products, Boston, MA) to allow coupling of Cy-5 dyes adjacent to the horseradish peroxidase-conjugated secondary antibody (tyramide is activated by horseradish peroxidase, and the activated form interacts covalently with adjacent protein molecules). The emission peak of Cy-5 may fall outside the tissue autofluorescence spectrum, thus minimizing background fluorescence for more accurate quantification of signal. The slides were then stained with the DNA staining dye 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, mounted with an antifade medium containing 0.6% n-propyl gallate in glycerol, and covered with a cover slip.

[0090] The slides may then be mounted (*e.g.*, using ImmunoMount; Shandon, Pittsburgh, PA) and analyzed by use of a conventional four-point scoring system for each biomarker (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining). Slides may be read by one or two observers who may be blinded to the outcome data for each slide.

[0091] The AQUA system may be used for automated image acquisition and analysis. Images of the tissue microarray core sections (histospots) and cell lines may be captured with a microscope and analyzed with using AQUA software (Camp *et al.*, *Nat Med* 2002;8:1323-7). For each histospot, areas of tumor are distinguished from stromal elements by creating an epithelial tumor mask from the antikeratin protein signal, which may be visualized via a Alexa 546 fluorophore. The tumor mask may be determined by gating the pixels in this image, in which an intensity threshold is set by visual inspection of histospots, and each pixel was recorded as "on" (tumor) or "off" (nontumor) by the software on the basis of the threshold. The DAPI image, which was used to identify the nuclei, may be subjected to a rapid exponential subtraction algorithm that can improve signal-to-noise ratio by subtracting the out-of-focus image from the in-focus image. After application of the rapid exponential subtraction algorithm, the signal intensity of the target antigen (*e.g.*, CCNB1, PR, BCL2, PAI1, ER, GATA3, or EIG121), which was acquired under a Cy5 signal, was scored on a scale of 0-255. The AQUA score within the subcellular compartments (*i.e.*, nucleus and membrane) may be calculated by dividing the signal intensity by the area of the specified compartment. The AQUA score for the cell lines may be determined by dividing the signal intensity by the total area under the tumor mask.

[0092] In addition to the AQUA system, virtually any other method which can quantify the expression of CCNB1, PR, BCL2, PAI1, ER, GATA3, or EIG121 may be used

in conjunction with the methods of the present invention. For example, a variety of tissue arrays may be used to quantify, *e.g.*, via immunohistochemistry, one or more or all of CCNB1, PR, BCL2, PAI1, ER, GATA3, and/or EIG121.

5 Predicting recurrence risk and likelihood of response to therapy

Algorithm to generate a cancer recurrence score

[0093] The inventors have identified panels of protein markers, the expression levels of these markers in combination with algorithms are useful in predicting recurrence risk of breast cancer patients after an anti-estrogen drug therapy and/or responsiveness of breast cancer patients to an anti-estrogen drug therapy. In some embodiments, the protein expression levels of CCNB1 and PAI1 are used in the prediction. In some embodiments, the protein expression levels of CCNB1, PR and BCL2 are used in the prediction. In some embodiments, the protein expression levels of CCNB1, PR, BCL2, and PAI1 are used in the prediction. In some embodiments, the protein expression levels of CCNB1, ER, PR, BCL2, and GATA3 are used in the prediction. In some embodiments, these markers are human proteins. For example, the marker proteins may be the expression products of the following nucleotides provided at GenBank: Accession Nos. NM_031966 (human CCNB1), NM_000633 (human BCL2), NM_002051 (human GATA3), NM_000125 (human ESR1), NM_000602 (human PAI1), and NM_000926 (human PR). In certain embodiments, expression levels are measured on a breast cancer sample which has been surgically removed or a breast cancer tissue biopsy. The breast cancer sample or tissue biopsy may be obtained from a subject prior to administration of an anti-estrogen drug to the subject.

[0094] Quantified protein expression data from patients with known treatment outcomes is analyzed using known programs and algorithms, and mathematical equations or models for calculating recurrence risk are generated and the thresholds (cutoff points) are defined to classify patients into high risk, moderate or middle risk, and low risk groups.

[0095] In one embodiment, a multivariate COXPH model is used as the prediction model. Four multiple-component classifiers, each in the form of a mathematical equation, are created based on the fitting of the multivariate COXPH models to the features using the entire training set. Each component in an equation is a protein that is weighted by the estimated logarithm of the hazard ratio derived from the COXPH modeling for recurrences versus non-recurrences. The mathematical equations calculate Risk Scores (RS) for each patient of the training set. The higher the RS, the higher the risk of cancer recurrence. The cutoff points

are defined by the lower and upper tertiles of the RS, classifying patients into three groups: the lowest risk (RS less than or equal to the lower tertile), the middle risk (RS higher than the lower tertile but less than the upper tertile), and the highest risk (RS higher than or equal to the upper tertile). The classifiers and the cutoff points may be cross-validated using patient data from an independent study. Kaplan-Meier survival analysis may be used to show that the three risk groups of the validation set are significantly different in recurrence-free survival (RFS) (e.g., $p < 0.01$ in log rank test), and/or the recurrence rate of the patients in the high risk group is significantly higher than that of the patients in the low risk group (e.g., $p < 0.001$, 0.0001, 0.00001, or 0.000001). As shown in the Examples described herein, patients in the lowest risk group have better recurrence-free survival (RFS), indicating better response to the treatment.

[0096] In some embodiments, the risk score (RS) of a patient equals to the sum of products, wherein each product is the expression level of each protein marker in the panel in the patient sample multiplied by a coefficient reflecting its relative intra-set contribution to the risk of cancer recurrence. The coefficient of each marker and the predetermined thresholds or cutoff points for classifying the patient into a high risk, an intermediate risk and a low risk group are determined based on samples from patients with known outcomes from a certain treatment. For example, recurrence risk of a patient may be calculated using any of the mathematical equations and thresholds described in Example 1 for RPPA assay system. The coefficients and thresholds in the mathematical equation may vary if a different assay system is used, and may be established and validated using clinical samples for each assay system. For example, these parameters may be established and validated for using immunohistochemistry methods to measure protein expression levels of the markers. In some embodiments, the RS is calculated using an automated program in a computer.

[0097] In some embodiments, the expression level of a protein marker used in predicting the risk of cancer recurrence and responsiveness to a therapy is an average value, a median value, or a mean value of the expression level measured in the patient sample, such as a cancer or breast cancer tissue sample. In some embodiments, the expression level of a protein marker used in predicting the risk of cancer recurrence and responsiveness to a therapy is normalized using a reference level. In some embodiments, the normalized expression level of the marker gene is calculated as a ratio of or difference between the marker gene and reference expression levels, on the original or on a log scale, respectively.

[0098] The RS scores and the algorithms of the invention may be used to determine the likelihood of breast cancer recurrence or response to a treatment for breast cancer

patients. In some embodiments, the patient has been diagnosed with hormone dependent breast cancer. In some embodiments, the patient has been diagnosed with ER-positive breast cancer. In some embodiments, the patient has been diagnosed with lymph node-negative ER-positive breast cancer. In some embodiments, patient has been diagnosed with lymph node-positive ER-positive breast cancer. Types of breast cancer may be diagnosed using conventional criteria known in the art. See, e.g., Paik *et al.*, *N. Engl. J. Med.* 351:2817-26, 2004; and Costantino *et al.*, *N. Engl. J. Med.* 320:479-84, 1989. A breast cancer may be diagnosed as hormone dependent if a hormone receptor test indicates that the tumor has a positive score for estrogen receptor and/or progesterone receptor. For example, a breast cancer patient may be diagnosed as having ER-positive breast cancer if the tumor has ≥ 10 fmol/mg cytosol protein. Lymph node status (*i.e.*, positive or negative) may be diagnosed by histological assessment. In some embodiments, the method described herein is used for predicting 5-year relapse, 10-year relapse, 15-year relapse, and/or 20-year relapse.

[0099] The methods described herein may also be automated in whole or in part. For example, the expression levels of each marker in a panel are entered into a computer or other automated machines for determining the risk score based on one or more of the algorithms described herein and/or predicting recurrence risk and likelihood of response to an anti-estrogen drug therapy for a patient. A report summarizing the result of the determination is generated from the computer or other automated machines. The report may include results of recurrence risk scores, classifying the patient as having high, middle, or low risk of relapse, and/or treatment recommendations.

Treatment with anti-estrogen drugs

[0100] Recurrence risk scores (RSs) as determined by the method of the present invention, provide valuable tools for the practicing physician to make critical treatment decisions for breast cancer patients. For example, if the RS of a particular patient is low, the physician might decide that other therapies (such as chemotherapy) is not necessary after the anti-estrogen drug treatment in order to ensure long term survival of patient. If, on the other hand, the RS is determined to be high, the physician might decide other treatment option (such as, radiation therapy and/or chemotherapy) are necessary for treating the patient after the anti-estrogen treatment. Similarly, if the likelihood of response of a patient to an anti-estrogen drug therapy is high, a physician might decide to use the anti-estrogen drug as a treatment modality for the patient. If the likelihood of response of a patient to an anti-estrogen drug therapy is low, other, more effective, treatment modalities may be used to

combat cancer in that particular patient. For each model there are generally two cutoff points: lower and upper, which are the tertile values of the risk scores. RS falling in the first, second, or third tertile is defined as low, intermediate, or high RS, respectively.

[0101] Determining the RS for a particular patient by the method of the present invention will enable the physician to tailor the treatment of the patient such that the patient has the best chance of long term survival while unwanted side-effects are minimized. If a breast cancer patient is identified as having a low recurrence risk score or high likelihood of response to an anti-estrogen drug treatment, the patient may be treated with the anti-estrogen receptor drug.

[0102] Anti-estrogen drugs include drugs that are antagonists that inhibit the binding of estrogen to the estrogen receptor, inhibitors of estrogen biosynthesis, and inhibitors that block estrogen receptor signaling.

[0103] The most commonly used anti-estrogen drug is tamoxifen, which competes with estrogen for binding to estrogen receptors on tumors. Tamoxifen is currently used for the treatment of both early and advanced ER+ (estrogen receptor positive) breast cancer in pre- and post-menopausal women. It is also approved by the FDA for the prevention of breast cancer in women at high risk of developing the disease. Clinical studies have shown that the use of tamoxifen as an adjuvant therapy after surgery reduces the risk of cancer recurrence; however, the response of ER+ patients to this treatment varies.

[0104] Other anti-estrogen drugs include raloxifene, which, like tamoxifen, blocks the effect of estrogen on breast tissue and breast cancer; and toremifene citrate, which is closely related to tamoxifen, and may be an option for post menopausal women with metastatic breast cancer.

[0105] Anastrozole, an aromatase inhibitor, acts by preventing estrogen from activating its receptor, blocking an enzyme needed for production of estrogen. Anastrozole is currently an option for women whose advanced breast cancer continues to grow during or after tamoxifen treatment.

[0106] Megesterol acetate is typically used for hormonal treatment of advanced breast cancer, usually for women whose cancers fail to respond to tamoxifen.

[0107] Toremifene is another anti-estrogen closely related to tamoxifen. It may be used, *e.g.*, to treat postmenopausal women with metastatic breast cancer

[0108] Fulvestrant (also known as ICI 182,780) is an estrogen receptor antagonist that can reduce the number of estrogen receptors. It may be used to treat a breast cancer after prior treatment with tamoxifen.

[0109] Other therapies for treating breast cancer (such as chemotherapy, radiation therapy or radiotherapy, surgery, gene therapy, immunotherapy, *etc.*) are available and may be used in conjunction with the anti-estrogen drug after determining the recurrence risk of a breast cancer patient. As used herein, "in conjunction" includes simultaneous administration and/or administration at different times. Administration in conjunction also encompasses administration as a co-formulation (i.e., different drugs are present in the same composition) or administration as separate compositions, administration at different dosing frequencies or intervals, and administration using the same route or different routes.

10 C. Kits

[0110] The invention also provides kits for use in the methods described herein. Such kits may comprise at least one reagent specific for detecting and quantitating the expression level of a marker protein described herein, and may further include instructions for carrying out a method described herein.

15 [0111] In some embodiments, the kit comprises one or more antibodies that specific bind or recognize a protein marker selected from the group consisting of CCNB1, PR, BCL2, PAI1, ER, and GATA3. In some embodiments, the kit comprises an antibody that specifically bind to CCNB1 and an antibody that specifically binds to PAI1. In some
20 embodiments, the kit comprises an antibody that specifically bind to CCNB1, an antibody that specifically binds to PR, and an antibody that specifically binds to BCL2. In some embodiments, the kit comprises an antibody that specifically bind to CCNB1, an antibody that specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to PAI1. In some embodiments, the kit comprises an antibody that specifically bind to CCNB1, an antibody that specifically binds to ER, an antibody that
25 specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to GATA3. In some embodiments, the antibodies bind to human marker proteins. In some embodiments, the kit further comprises a composition for detecting the binding of the antibodies to proteins, such as one or more secondary antibodies that specifically bind to the antibodies that bind to the marker proteins. In some embodiments,
30 the secondary antibodies are labeled. In some embodiments, the kit further comprises reagents for sample preparation and detecting antibody bindings. In some embodiments, the kit may further comprise a computer program a computer-readable medium containing a program for calculating the recurrence risk score or likelihood of response to treatment for patients. In some embodiments, the kit may further comprise one or more positive and

negative control antibodies for the immunoassay. In some embodiments, the kit further comprises a package insert providing instructions for measuring the expression levels of one or more marker proteins and determining the recurrence risk and likelihood of response to treatment. In some embodiments, the package insert further provides instructions for using a digital imaging system to detect binding of the antibodies to the protein markers. Mathematical algorithms used to calculating the recurrence risk and likelihood of response to treatment may also be included in the package insert. In various embodiments, the kit may comprise an ELISA test or a lateral flow assay.

[0112] The following are examples of the methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

EXAMPLES

Example 1. Identification of protein markers for predicting recurrence risk of tamoxifen-treated ER⁺ breast cancers

[0113] Studies were carried out to identify classifiers for the prediction of the 5-year recurrence risk in patients with ER⁺ breast cancers treated with tamoxifen. The four main steps were used to developing the classifiers: (1) selecting features, (2) selecting a prediction model, (3) fitting the prediction models to training data, and (4) validating the models with independent data.

[0114] Primary breast tissues were collected from breast cancers at breast surgery under an Institutional Review Board-approved protocol. Each tumor was sectioned and frozen in liquid nitrogen within one hour of surgical excision after review of the tumor and a frozen section by a pathologist. These tumors were ER-positive and the patients were treated with Tamoxifen after surgery. Frozen tumor tissue (≤ 10 mg) was homogenized after macrodissection in lysis buffer at 40mg/ml by PowerGen polytron homogenizer (Fisher Scientific, Hampton, NH) and the concentration of the protein lysates corrected to 1.33 mg/mL. After centrifugation, post-nuclear detergent lysates (3 parts) were boiled with a solution (1 part) of 4xSDS (90%)/B mercapto-ethanol (10%). Five serial twofold dilutions were performed in lysis buffer containing 1% SDS (dilution buffer). The diluted lysates were spotted on nitrocellulose-coated FAST slides (Whatman, Schleicher & Schuell BioScience, Inc., Keene, NH) by a robotic GeneTAC (Genomic Solutions, Inc., Ann Arbor, MI) G3 arrayer or an Aushon Biosystems (Burlington, MA) 2470 arrayer. The DAKO (Carpinteria, CA) catalyzed signal amplification system was used for antibody blotting. Each slide was

incubated with a primary antibody (BCL2, CCNB1, ER, GATA3, PAI1, or PR) in the appropriate dilution. The antibody directed to BCL2 (Catalog No. M0887) was purchased from Dako (Carpinteria, CA, USA); the antibody directed to ER alpha (Catalog No. RM-9101-S) was purchased from Lab Vision Corporation (Fremont, CA, USA); the antibody
5 directed to CCNB1 (Catalog No. 1495-1) or PR (Catalog No. 1483-1) was purchased from Epitomics, Inc (Burlingame, CA, USA); and the antibody directed to GATA3 (Catalog No. 558686) or PAI1 (Catalog No. 612024) was from BD Biosciences (San Jose, CA, USA). The signal was captured by biotin-conjugated secondary antibody and amplified by tyramide deposition. The analyte was detected by avidin-conjugated peroxidase reactive to its substrate
10 chromogen diaminobenzidine (DAB). Subsequently, the slides were individually scanned, analyzed, and quantitated using MicroVigene software (VigeneTech Inc., North Billerica, MA). The signal intensity data from MicroVigene were processed by the R package SuperCurve (version 1.4), available at bioinformatics.mdanderson.org/OOMPA, which generated a relative log2 concentration for each protein.

15 **[0115]** The features were selected by two procedures: the statistically robust procedure and the biological hypothesis-driven procedure. The statistically robust procedure includes Monte Carlo (MC) re-sampling and Cox Proportional Hazards (COXPH) modeling: 75% of the patients were sampled from a training set ($n = 197$) 100 times with replacement to create 100 resampled datasets. Based on each resampled dataset, a univariate COXPH model
20 was used to identify proteins significantly associated with recurrence-free survival (RFS) ($p < 0.05$); then a multivariate RFS COXPH model was fitted to these significant proteins and the informative proteins were picked up by using AIC (Akaike's information criterion) stepwise selection on the model. The informative proteins identified from all the 100 resampled datasets were pooled and the most frequently occurring proteins were chosen from the pool
25 by a cutoff frequency of at least 60. Furthermore, a multivariate RFS COXPH model was fitted to the most frequently occurring proteins and the protein features were selected by AIC stepwise selection. A 2-protein feature consisting of CCNB1 and PAI1 was defined by the statistically robust procedure. The biological hypothesis-driven procedure selected two potential marker sets consisting of 5 (CCNB1, ER, PR, BCL2, and GATA3) and 7 (CCNB1,
30 ER, PR, GATA3, BCL2, EIG121, and PAI1) proteins, respectively. Based on the results of the COXPH modeling and AIC stepwise selection on the two hypothesis-driven marker sets, 3-protein (CCNB1, PR, and BCL2), 4-protein (CCNB1, PR, BCL2, and PAI1), and 5-protein (CCNB1, ER, PR, BCL2, and GATA3.BD) features were defined.

[0116] The prediction model is a multivariate COXPH model. Four multiple-component classifiers, each in the form of a mathematical equation, were created based on the fitting of the multivariate COXPH models to the features using the entire training set. Each component in an equation is a protein that is weighted by the estimated logarithm of the hazard ratio derived from the COXPH modeling for recurrences versus non-recurrences. The mathematical equations calculate Risk Scores (RS) for each patient of the training set. Higher RS indicates higher risk of recurrence. The cutoff points are defined by the lower and upper tertiles of the RS, classifying patients into three groups: the lowest risk (RS less than or equal to the lower tertile), the middle risk (RS higher than the lower tertile but less than the upper tertile), and the highest risk (RS higher than or equal to the upper tertile). Patients in the high risk groups are more likely to develop disease relapse than those in the middle and low risk groups. The classifiers and the cutoff points were cross-validated using an independent study containing 77 ER⁺ breast cancer patients treated with tamoxifen. Kaplan-Meier survival analysis indicated that the three risk groups of the validation set were significantly different in RFS (log rank test $p < 0.01$) and that the patients in the lowest risk group had better RFS. The recurrence rate of the highest risk group was significantly higher than that of the lowest risk group ($p < 10^{-6}$).

[0117] The multiple-components mathematical equations (classifiers) and their cutoff points for each of the marker panels were generated based on the data from patients.

Model 1: $0.466 \times \text{"CCNB1"} - 0.183 \times \text{"PR"} - 0.199 \times \text{"BCL2"}$

Cutoff points for model 1: lower: -0.7299977; upper: -0.1393642

Model 2: $0.367 \times \text{"CCNB1"} + 0.199 \times \text{"PAI1"}$

Cutoff points for model 2: lower: -0.4238341847; upper: 0.0005819119

Model 3: $0.442 \times \text{"CCNB1"} - 0.161 \times \text{"PR"} - 0.240 \times \text{"BCL2"} + 0.213 \times \text{"PAI1"}$

Cutoff points for model 3: lower: -0.7997882; upper: -0.1449444

Model 4: $0.4175 \times \text{"CCNB1"} + 0.0898 \times \text{"ER"} - 0.191 \times \text{"PR"} - 0.2281 \times \text{"BCL2"} - 0.0733 \times \text{"GATA3"}$

Cutoff points for model 4: lower: -0.60177969; upper: -0.06292317

[0118] The protein symbols in the equations represent the protein expression levels of the respective proteins. Using protein markers' expression levels, each equation independently calculates Risk Score (RS) for each patient who is then classified into the lowest risk (RS smaller than or equal to the lower cutoff point), the middle risk (RS higher

than the lower cutoff point but less than the upper cutoff point), or the highest risk (RS higher than or equal to the upper cutoff point) group based on the cutoff points.

Example 2. Use of the marker panels to predict recurrence risk of ER⁺ breast cancer patients treated with tamoxifen or response to tamoxifen treatment

[0119] The protein marker panels were validated by an independent validation set containing RPPA (reverse phase protein array) data for 77 ER-positive breast cancer patients treated with Tamoxifen. The RS calculated by the four marker panels described in Example 1 showed strong prediction of 5-year recurrence, which was presented by Receiver Operating Characteristic (ROC) curves (Figure 1). ROC curve plots true positives (sensitivity) versus false positives (1-specificity) describes the performance of a model across the entire range of classification thresholds and is a direct way to cost/benefit analysis of diagnostic decision making. The performance can be assessed by measuring the area under the ROC curve. Higher AUC indicates better performance. An AUC value of 0.5 indicates random performance; and an AUC value of 1.0 indicates perfect performance. The AUCs were 0.74, 0.77, 0.76, and 0.76 for the 2-protein model (CCNB1 and PAI1), 3-protein model (CCNB1, PR and BCL2), 4-protein model (CCNB1, PR, BCL2, and PAI1), and 5-protein model (CCNB1, ER, PR, BCL2, and GATA3), respectively (FIGS. 1A-D). In addition, the RS was able to categorize the 77 patients into three risk groups. Kaplan-Meier Survival analysis and log rank test indicated that the three risk groups were significantly different in RFS (FIGS. 2A-D) using the 2-, 3-, 4-, and 5-protein models, respectively.

[0120] The 3-protein model (CCNB1, PR and BCL2) was also validated using RPPA data from lymph node-negative, estrogen receptor positive breast cancer patients treated with tamoxifen. Kaplan-Meier Survival analysis and log rank test indicated that the three risk groups were significantly different in RFS (FIG. 3).

[0121] Four Kaplan-Meier overall survival curves were obtained for the 2-protein model, 3-protein model, 4-protein model, and 5-protein model, respectively, using an independent validation set, TCGA breast cancer set. Only ER+/PR+ patients were used and the overall survival was censored at 5 years (60 months). The risk groups were observed to be significantly different in overall survival (FIGS. 4A-D).

[0122] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention.

CLAIMS

What is claimed is:

1. A method for determining the likelihood of breast cancer recurrence after treatment or response to a treatment with an anti-estrogen drug in an individual, said method
5 comprising:

(a) measuring the protein expression levels of a panel of markers in a biological sample containing breast cancer cells obtained from the individual, wherein the panel of markers comprises:

(i) CCNB1 and PAI1;

10 (ii) CCNB1, PR and BCL2;

(iii) CCNB1, PR, BCL2, and PAI1, or

(iv) CCNB1, PR, BCL2, ER, and GATA3;

(b) calculating the recurrence risk score (RS) for said individual based on the expression levels of the markers in the panel; and

15 (c) using the RS to determine the likelihood of breast cancer recurrence or response to the treatment.

2. The method of claim 1, wherein the RS is the sum of the measured expression level of each marker in the panel in step (a) multiplied by a coefficient reflecting the relative
20 contribution of the expression level of the marker to cancer recurrence or response to the treatment.

3. The method of claim 2, wherein the RS value higher than a predetermined threshold indicates an increased likelihood of breast cancer recurrence; or wherein the RS
25 value lower than a predetermined threshold indicates a decreased likelihood of breast cancer recurrence.

4. The method of claim 3, wherein the coefficients for CCNB1, PAI1, and ER have positive values, and the coefficients for PR, BCL2, and GATA3 have negative values.

30 5. The method of claim 2, wherein the coefficient for each marker in the panel is determined based on the protein expression levels in clinical samples from individuals whose risk of breast cancer recurrence or responses to the treatment are known.

6. The method of claim 1, further comprising a step of generating a report summarizing the result of said determination.

7. The method of claim 6, wherein the report further comprises information indicating whether said individual should receive treatment with said anti-estrogen drug alone, any other therapy alone, or said anti-estrogen drug plus any other therapy.

8. The method of claim 1, wherein the individual is human.

9. The method of claim 8, wherein said biological sample is a tumor tissue obtained from said human individual.

10. The method of claim 9, wherein said biological sample is a fixed, paraffin-embedded tissue section.

11. The method of claim 9, wherein said biological sample is a fresh frozen tissue section.

12. The method of claim 8, wherein the individual has a hormone dependent breast cancer.

13. The method of claim 8, wherein the individual has an ER-positive breast cancer.

14. The method of claim 8, wherein the method comprises determining 5-year relapse of lymph node-negative or lymph node-positive ER-positive breast cancer.

15. The method of claim 1, wherein the anti-estrogen drug is an estrogen receptor antagonist in breast tissue.

16. The method of claim 1, wherein the anti-estrogen drug is an aromatase inhibitor.

17. The method of claim 1, wherein the anti-estrogen drug is a steroid sulfatase inhibitor.

18. The method of claim 1, wherein the anti-estrogen drug is selected from the group consisting of tamoxifen, toremifene, fulvestrant, anastrozole, and megesterol acetate.

19. The method of claim 1, wherein the protein expression levels are measured using reverse phase protein array.

20. The method of claim 1, wherein the protein expression levels are measured by an immunoassay method

21. The method of claim 20, wherein the immunoassay method is selected from the group consisting of an immunohistochemistry assay, an automated quantitative analysis (AQUA) system assay, a lateral flow assay, a chemiluminescent labeled sandwich assay, and an enzyme-linked immunosorbent assay (ELISA).

22. A kit for determining the likelihood of breast cancer recurrence after treatment or response to a treatment with an anti-estrogen drug in an individual, comprising a panel of antibodies selected from the group consisting of:

1) an antibody that specifically binds to CCNB1, and an antibody that specifically binds to PAI1;

2) an antibody that specifically binds to CCNB1, an antibody that specifically binds to PR, and an antibody that specifically binds to BCL2;

3) an antibody that specifically binds to CCNB1, an antibody that specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to PAI1; and

4) an antibody that specifically binds to CCNB1, an antibody that specifically binds to ER, an antibody that specifically binds to PR, an antibody that specifically binds to BCL2, and an antibody that specifically binds to GATA3.

23. The kit of claim 22, wherein the antibodies are conjugated to a label.

24. The kit of claim 22, wherein the label is a fluorophore or an enzyme.

25. The kit of claim 22, wherein each antibody is conjugated a different label.

26. The kit of claim 22, wherein the antibodies in the kit bind to human marker proteins.

27. The kit of claim 22, further comprising a package insert providing instructions for measuring the expression levels of the markers in a biological sample from the individual and/or determining the recurrence risk and likelihood of response to the treatment.

FIG. 1B

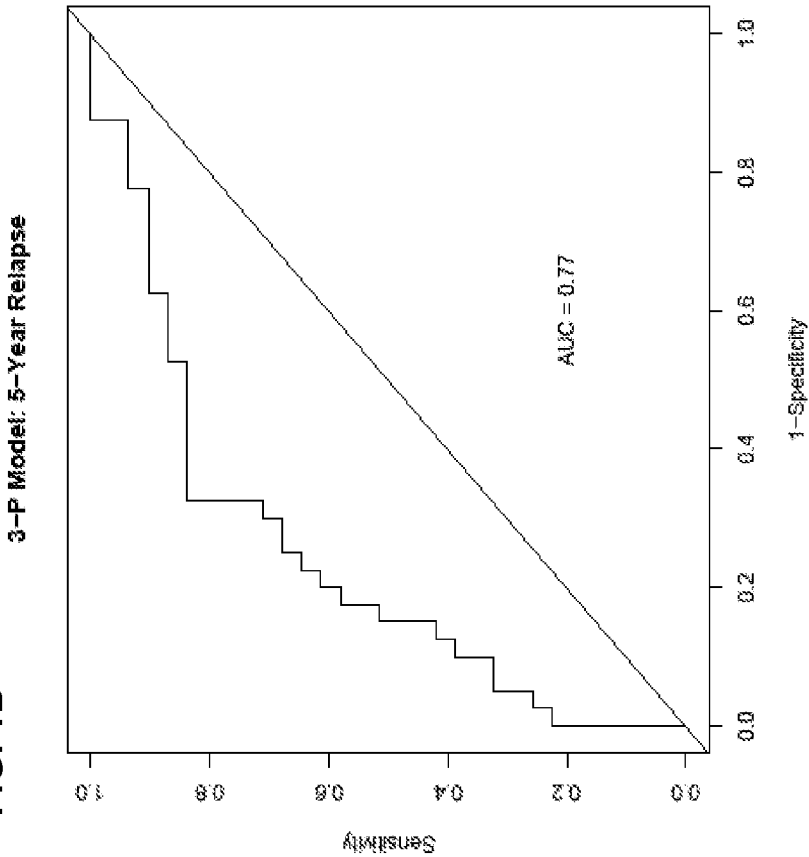


FIG. 1A

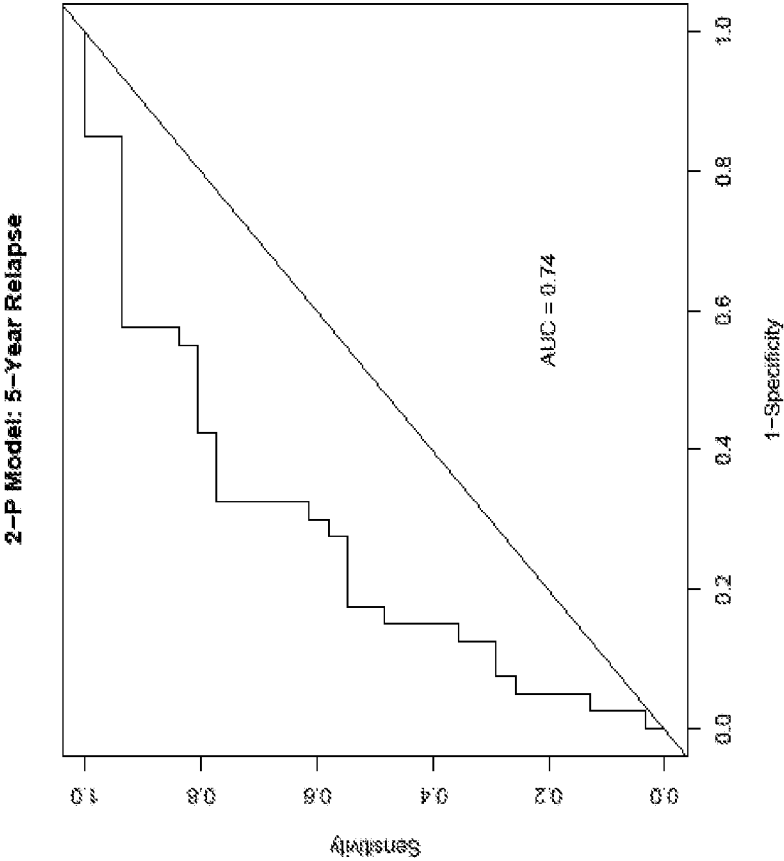


FIG. 1D

5-P Model: 5-Year Relapse

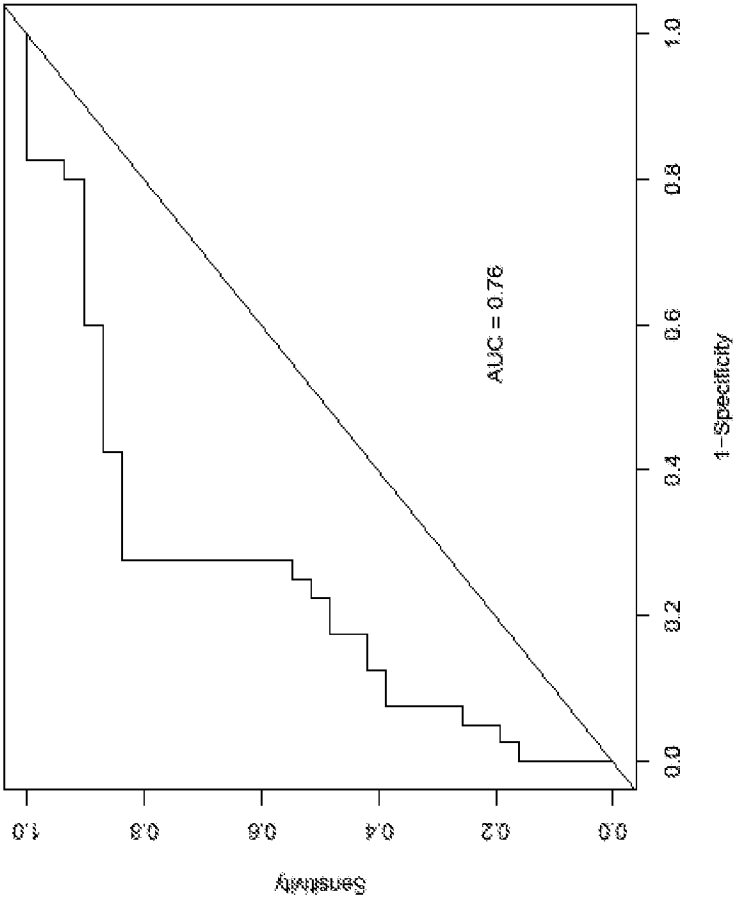


FIG. 1C

4-P Model: 5-Year Relapse

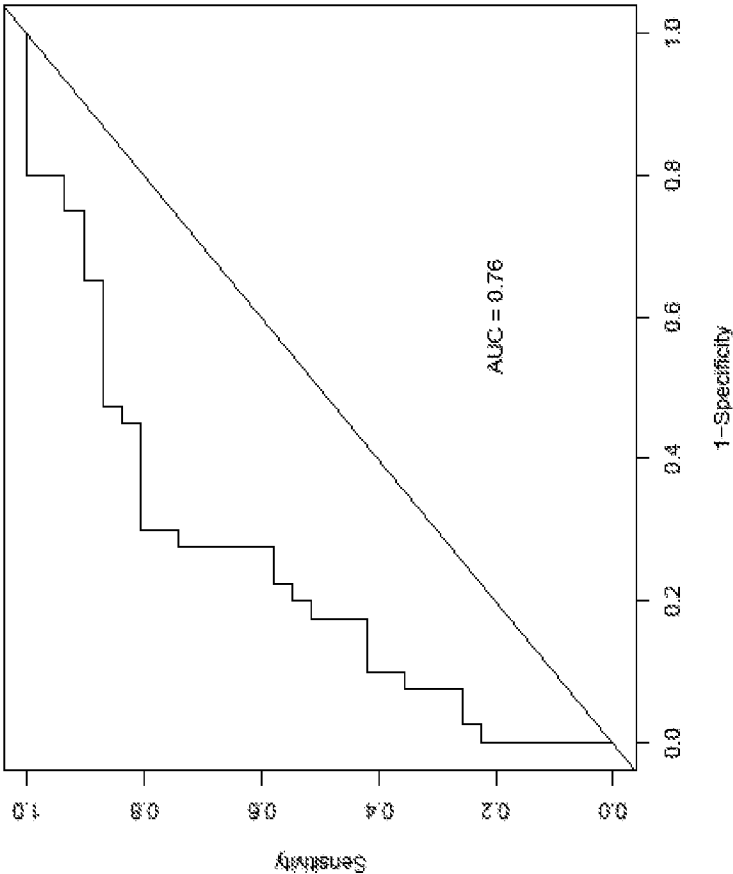


FIG. 2A

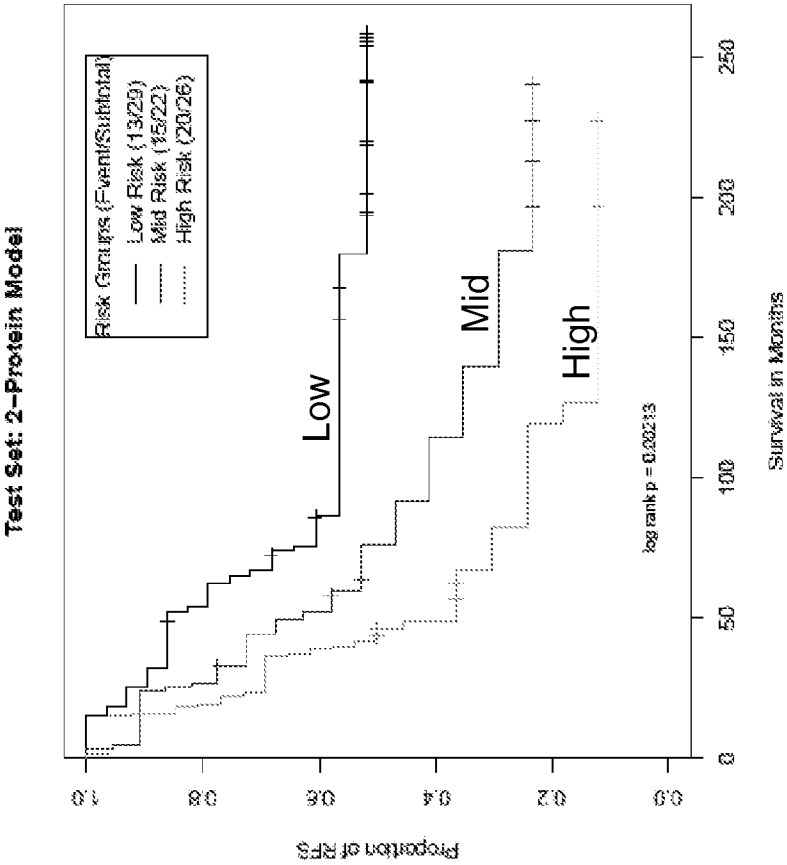


FIG. 2B

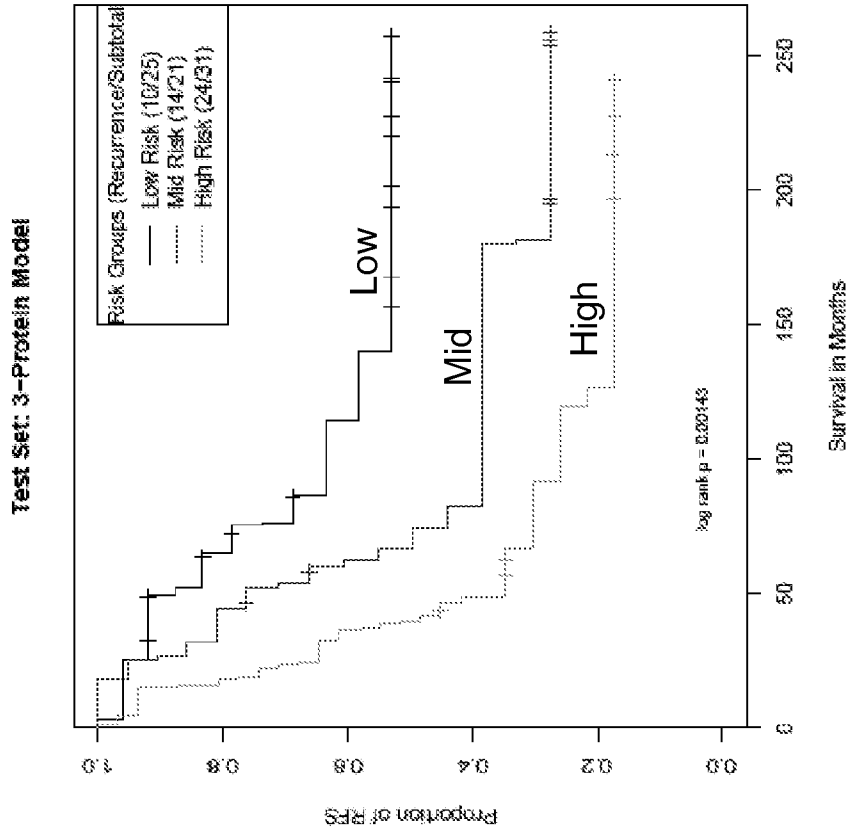


FIG. 2C

Test Set: 4-Protein Model

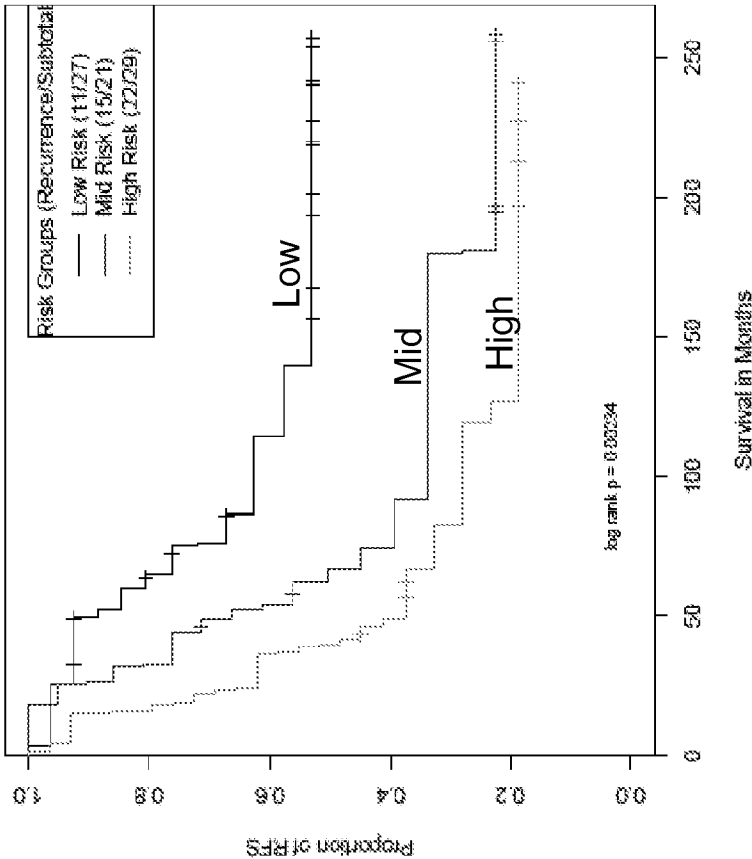


FIG. 2D

Test Set: 5-Protein Model

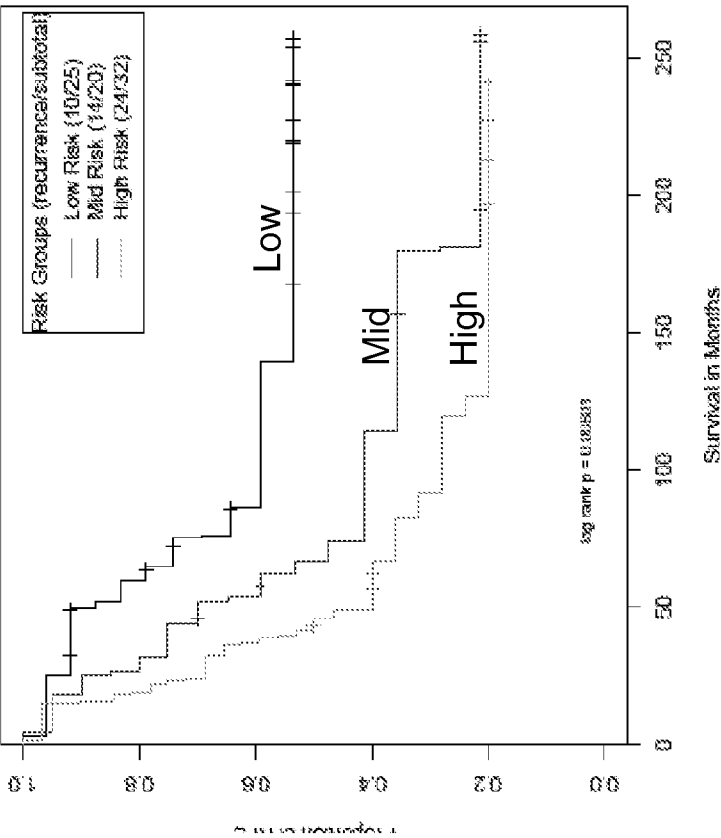


FIG. 3

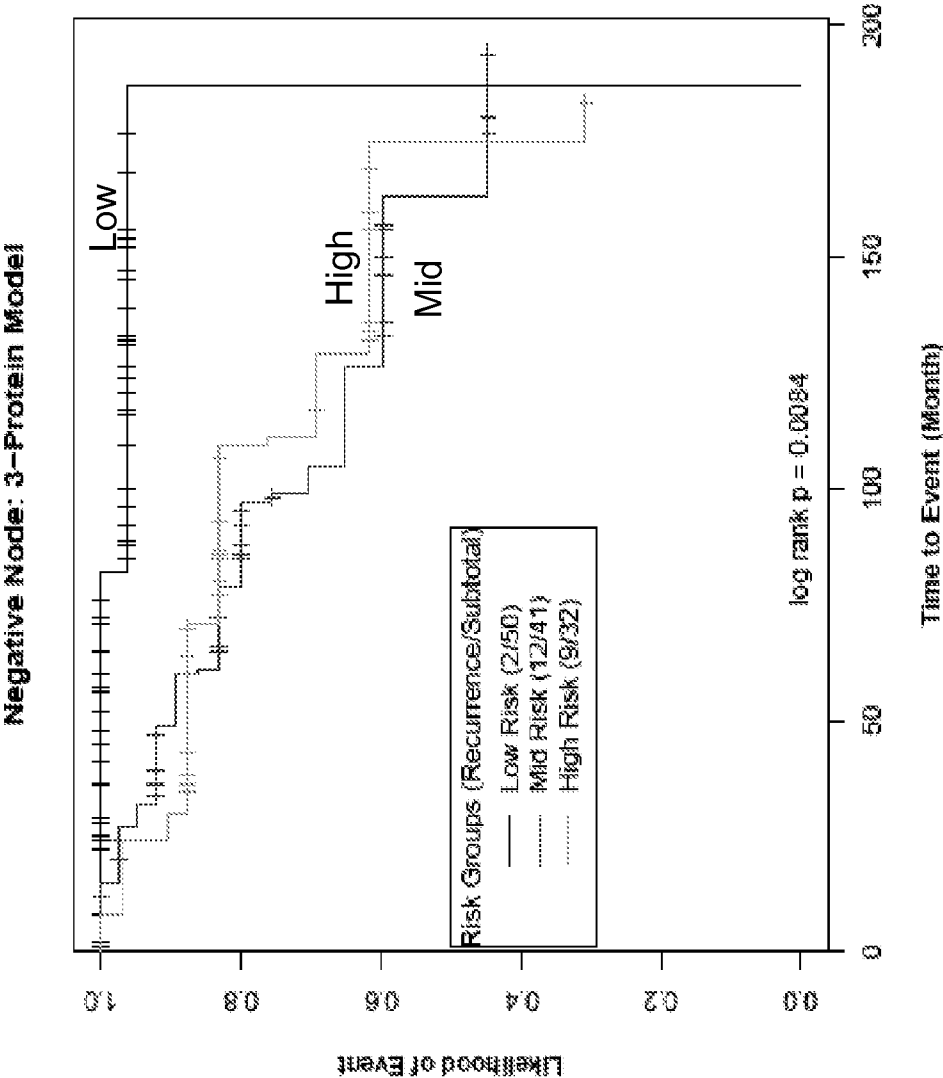


FIG. 4A: 2-protein model

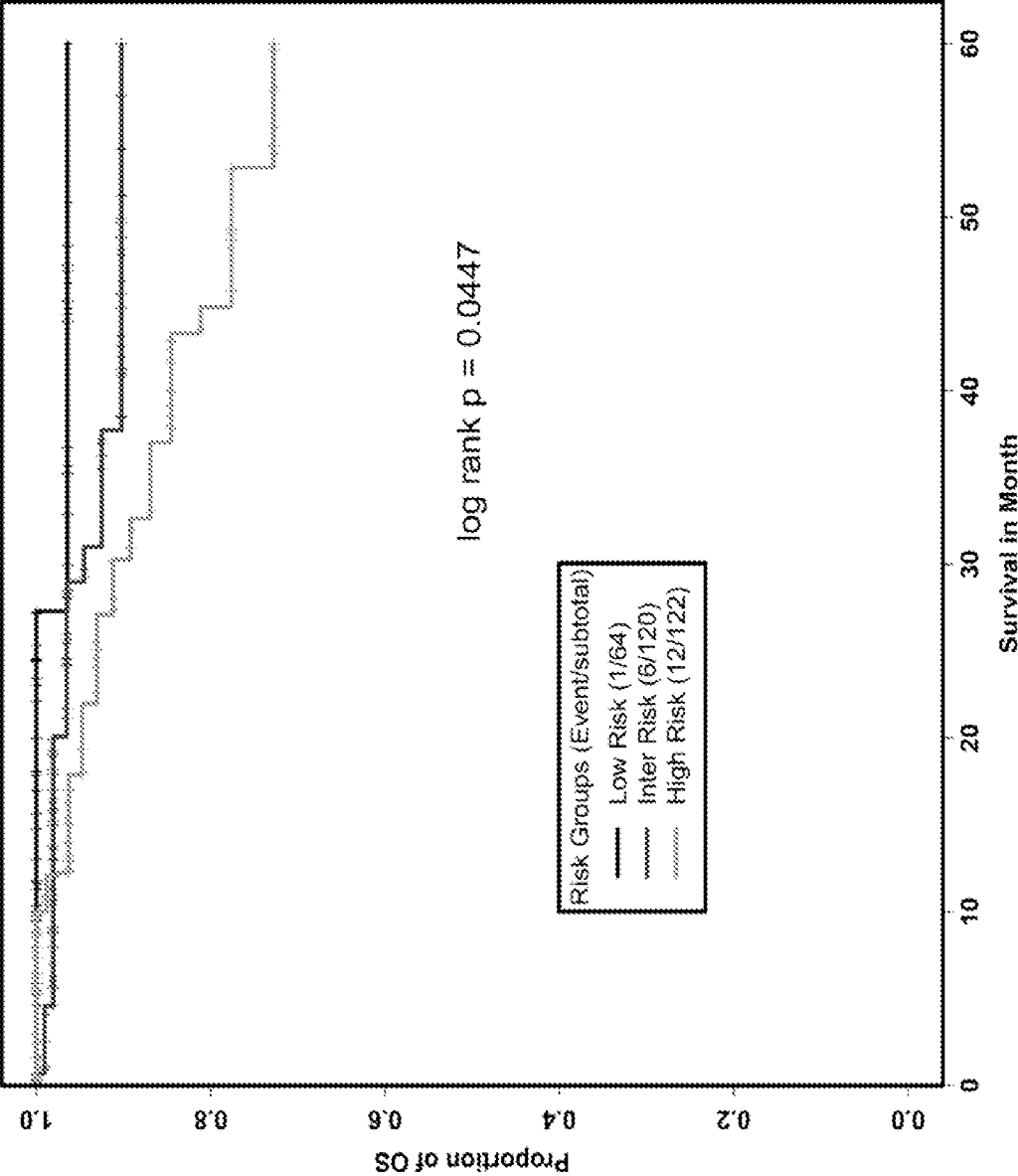


FIG. 4B: 3-protein model

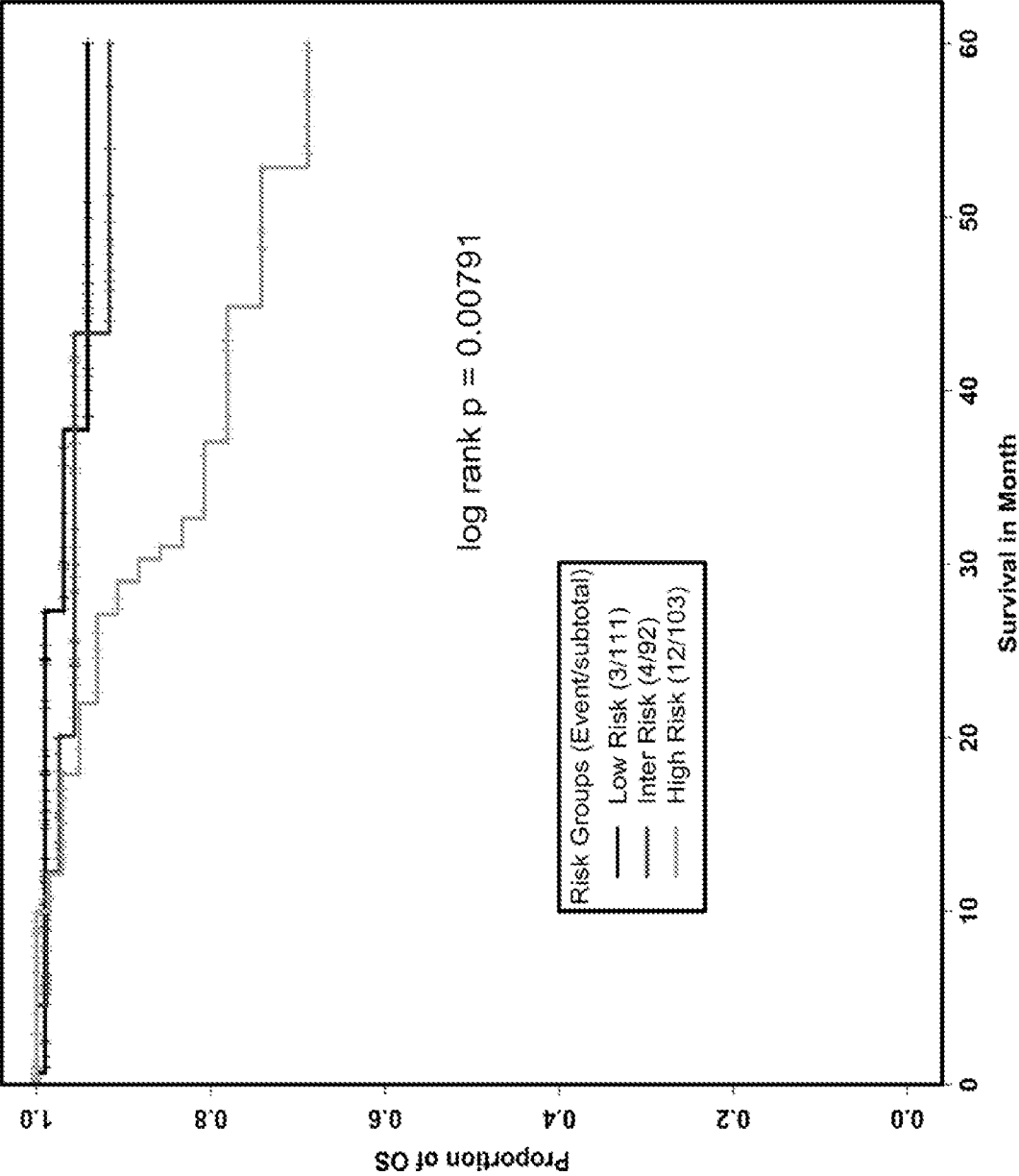


FIG. 4C: 4-protein model

