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(54) **Title**: METHODS AND MARKERS FOR PREDICTING RESPONSES TO CHEMOTHERAPY

(57) **Abstract**: The present invention provides methods for determining or predicting the sensitivity and/or resistance of breast tumors to taxane therapy, including ER- breast tumors that may otherwise be considered good candidates for taxane therapy. By discriminating taxane-resistant and taxane-sensitive tumors, even within the ER- population, the present invention allows a treating physician to select more effective chemotherapeutic regimens and/or spare the patient from unnecessary toxicity.

METHODS AND MARKERS FOR PREDICTING RESPONSES TO CHEMOTHERAPY

PRIORITY

[001] This Application claims priority to U.S. Provisional Application No. 61/187,108 filed June 15, 2009, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[002] The present invention relates to methods for predicting a tumor's response to therapy, including the identification of taxane-responsive tumors. In particular, the invention relates to markers and tests that are predictive of a breast cancer patient's response to taxane chemotherapy.

BACKGROUND

[003] Paclitaxel belongs to the taxane family of therapeutics, which have emerged as critically important drugs for breast cancer treatment. In addition to inhibiting cell growth by interfering with microtubule disassembly, its mechanism of action also includes induction of apoptosis.

[004] Besides being a key predictor for endocrine therapy response, Estrogen Receptor (ER) status also influences sensitivity of breast cancer to chemotherapeutic agents such as paclitaxel, with ER negative tumors being more responsive. See, Meihua Sui et al., Estrogen Receptor α Mediates Breast Cancer Cell Resistance to Paclitaxel Through Inhibition of Apoptotic Cell Death. *Cancer Res.* 67(11):5337-5344 (2007). However, patient responses to chemotherapy remain variable even within the ER- population. Thus, methods and markers for distinguishing drug-sensitive and drug-resistant tumors are needed, including with respect to ER negative tumors.

SUMMARY OF THE INVENTION

[005] The present invention provides methods for determining or predicting the sensitivity and/or resistance of tumors to therapy, including taxane therapy. The invention provides assays and markers to help select optimal therapy for ER- breast tumors, including assays and markers for predicting the efficacy of taxane therapy. By discriminating drug-

resistant and drug-sensitive tumors, even within the ER- population of breast tumors, the present invention allows a treating physician to select more effective therapeutic regimens and/or spare the patient from unnecessary toxicity as a result of ineffective agents.

[006] The method of the invention comprises determining an estrogen receptor (ER) status for the tumor, and determining *in vitro* responsiveness of the malignant cells to taxane chemotherapy; and/or determining the presence, absence, or level of one or more markers indicative of apoptosis. Such markers are described in detail herein.

[007] *In vitro* chemosensitivity (or responsiveness) in some embodiments comprises preparing a cell culture from the tumor that is enriched for malignant cells, and testing the malignant cells for an *in vitro* response to paclitaxel or similar agent, as well as other candidate therapeutic agents. Generally, where the tumor is determined to be ER-, and the cultured malignant cells are determined to be responsive to the taxane *in vitro*, the tumor is scored as being sensitive to taxane chemotherapy. Alternatively, the tumor is determined to be ER+, and/or determined to be non-responsive to the taxane *in vitro*, and thereby scored as being resistant to taxane therapy.

[008] In some embodiments, the presence, absence, or level of one or more markers of apoptosis is determined, so as to add further predictive value. For example, malignant cells may be cultured as described herein, and after contact with paclitaxel or similar agent, the presence or absence of molecular markers indicative of apoptosis are determined. Such markers include caspase-3 cleavage and PARP cleavage. Alternatively still, the presence or absence of an apoptosis gene expression or proteomic signature may be determined in gene expression or proteomic profiles prepared from tumor specimens (or cultures derived therefrom). The gene expression or proteomic signature generally comprises the level of expression indicative of a responsive or sensitive cell sample, for three or more genes/proteins selected from AIF1, BAD, BCL2, BID, BIRC5, CASP1, CASP6, CASP7, COX4I1, CYCS, PARP12, and PARP4.

DESCRIPTION OF THE FIGURES

[009] **Figure 1a** shows a dose response curve for 10 ER+ breast cancer cell lines and 15 ER- breast cancer cell lines in response to paclitaxel in an in vitro chemoresponse assay. **Figure 1b** and **c** show pacitaxel responsiveness based on ER status in these cell lines.

[010] **Figure 2a** shows a dose response curve for 12 breast cancer primary cultures (A-L). **Figure 2b** shows the primary cultures' responsiveness based on ER status. **Figure 2c** shows the ERα status of each of the primary cultures by western blot.

[011] **Figure 3** (**a** and **b**) shows the activation of the apoptosis pathway by the presence of cleaved PARP and cleaved caspase-3 in response to paclitaxel in ER- cell lines. The apoptotic pathways are not activated by pacliatxel in ER+ cell lines.

[012] **Figure 4** shows the activation of the apoptosis pathway through the induction of cleaved caspase-3 in response to paclitaxel in ER- primary cultures. The activation of the apoptotic pathway correlates with in vitro responsiveness. Cleaved caspase-3 is determined by in cell western analysis in 12 breast cancer primary cultures (A-L).

DETAILED DESCRIPTION OF THE INVENTION

[013] The present invention provides methods for predicting the sensitivity and/or resistance of tumors to therapy, including with respect to ER- breast tumors. By discriminating drug-resistant and drug-sensitive tumors, even within the ER- population, the present invention allows a treating physician to select more effective therapeutic regimens and/or spare the patient from unnecessary toxicity that may occur through administration of ineffective chemotherapy.

[014] The method of the invention comprises determining an estrogen receptor (ER) status for the tumor, and determining an *in vitro* chemoresponse of the tumor to chemotherapeutic agents; and/or determining the presence, absence, or level of one or more markers indicative of apoptosis.

Patients and ER Status

[015] The present invention involves testing for the presence or absence of markers indicative of a breast tumor's response to chemotherapy. The tumor specimen may be obtained from the patient by surgery, or may be obtained by biopsy, such as a fine needle biopsy or other procedure prior to the selection/initiation of neoadjuvant therapy. In certain embodiments, the cancer is breast cancer, including preoperative or post-operative breast cancer. In certain embodiments, the patient has not undergone treatment to remove the breast tumor, and therefore is a candidate for neoadjuvant therapy.

[016] The cancer may be primary or recurrent, and may be of any stage (e.g., Stage I, II, III, or IV or an equivalent of other staging system), and/or histology.

[017] The breast tumors may be classified into estrogen receptor positive (ER+) and negative (ER-) subtypes by any suitable method, including immunohistochemistry or other immunoassay with antibody against ER. Alternatively, ER status may be determined by ER+ or ER- gene expression signatures, as described, for example, in Gruvberger, S. et al., (2001) Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer Res.*, 61, 5979–5984; West, M. et al. (2001) Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc. Natl Acad. Sci. USA*, 98, 11462–11467; and Kun Yu et al., Classifying the estrogen receptor status of breast cancers by expression profiles reveals a poor prognosis subpopulation exhibiting high expression of the ERBB2 receptor, *Human Molecular Genetics* 12(24):3245-3258 (2003).

[018] Particularly where breast tumors are ER negative, and therefore considered likely to respond to chemotherapy (conventionally), in accordance with the invention, additional assays are used to better discriminate drug-sensitive and drug-resistant cells, to thereby select optimum therapy and avoid unnecessary toxicity. In such embodiments, the present invention provides a method of identifying a taxane-senstive breast tumor.

Chemosensitivity assay

[019] The invention may comprise conducting chemoresponse testing on the ER- or ER+ tumor samples. Chemoresponse testing may be conducted as described in U.S. Patent Nos. 5,728,541, 6,900,027, 6,887,680, 6,933,129, 6,416,967, 7,112,415, and 7,314,731 (all of which are hereby incorporated by reference in their entireties). The chemoresponse method may further employ the variations described in US Published Patent Application Nos. 2007/0059821 and 2008/0085519, both of which are hereby incorporated by reference in their entireties.

[020] Briefly, cohesive multicellular particulates (explants) are prepared from a patient's tissue sample (e.g., a biopsy sample) using mechanical fragmentation. This mechanical fragmentation of the explant may take place in a medium substantially free of enzymes that are capable of digesting the explant. However, in some embodiments, limited enzymatic treatment may be conducted. Generally, the tissue sample is systematically minced using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion creates smooth cut edges on the resulting tissue multicellular particulates. The tumor particulates each measure from about 0.25 to about 1.5 mm^3, for example, about 1 mm^3.

[021] After the tissue sample has been minced, the particles are plated in culture flasks (e.g., about 5 to 25 explants per flask). For example, about 9 explants may be plated per T-

25 flask, or about 20 particulates may be plated per T-75 flask. For purposes of illustration, the explants may be evenly distributed across the bottom surface of the flask, followed by initial inversion for about 10-15 minutes. The flask may then be placed in a non-inverted position in a 37°C CO_2 incubator for about 5-10 minutes. Flasks are checked regularly for growth and contamination. Over a period of days to a few weeks a cell monolayer will form. Further, it is believed (without any intention of being bound by the theory) that tumor cells grow out from the multicellular explant prior to stromal cells. Thus, by initially maintaining the tissue cells within the explant and removing the explant at a predetermined time (e.g., at about 10 to about 50 percent confluency, or at about 15 to about 25 percent confluency), growth of the tumor cells (as opposed to stromal cells) into a monolayer is facilitated. Further, in certain embodiments, the tumor explant may be agitated to substantially release tumor cells from the tumor explant, and the released cells cultured to produce a cell culture monolayer. The use of this procedure to form a cell culture monolayer helps maximize the growth of representative tumor cells from the tissue sample.

[022] Prior to the chemotherapy assay, the growth of the cells may be monitored, and data from periodic counting may be used to determine growth rates. Monolayer growth rate and/or cellular morphology and/or epithelial character may be monitored using, for example, a phase-contrast inverted microscope. Generally, the monolayers are monitored to ensure that the cells are actively growing at the time the cells are suspended for drug exposure. Thus, the monolayers will be non-confluent when the cells are suspended for chemoresponse testing.

[023] A panel of active agents may then be screened using the cultured cells. Generally, the agents are tested against the cultured cells using plates such as microtiter plates. For the chemosensitivity assay, a reproducible number of cells is delivered to a plurality of wells on one or more plates, preferably with an even distribution of cells throughout the wells. For example, cell suspensions are generally formed from the monolayer cells before substantial phenotypic drift of the tumor cell population occurs. The cell suspensions may be, without limitation, about 4,000 to 12,000 cells/ml, or may be about 4,000 to 9,000 cells/ml, or about 7,000 to 9,000 cells/ml. The individual wells for chemoresponse testing are inoculated with the cell suspension, with each well or "segregated site" containing about 10^2 to 10^4 cells. The cells are generally cultured in the segregated sites for about 4 to about 30 hours prior to contact with chemotherapeutic agent.

[024] Each test well is then contacted with at least one pharmaceutical agent, or a sequence or combination of agents. In addition to at least one taxane (e.g., paclitaxel and/or docetaxel), the panel of chemotherapeutic agents may comprise at least one agent selected

from a platinum-based drug, a nitrogen mustard, a kinase inhibitor, a pyrimidine analog, a podophyllotoxin, an anthracycline, a monoclonal antibody, and a topoisomerase I inhibitor. For example, the panel may comprise 1, 2, 3, 4, or 5 agents selected from bevacizumab, capecitabine, carboplatin, cecetuximab, cisplatin, cyclophosphamide, doxorubicin, epirubicin, etoposide, 5-fluorouracil, gemcitabine, irinotecan, oxaliplatin, panitumumab, tamoxifen, topotecan, and trastuzumab, in addition to other potential agents for treatment. In certain embodiments, the chemoresponse testing includes one or more combination treatments, such combination treatments including one or more agents described above. Generally, each agent in the panel is tested in the chemoresponse assay at a plurality of concentrations representing a range of expected extracellular fluid concentrations upon therapy.

[025] The efficacy of each agent in the panel is determined against the patient's cultured cells, by determining the viability of the cells (e.g., number of viable cells). For example, at predetermined intervals before, simultaneously with, or beginning immediately after, contact with each agent or combination, an automated cell imaging system may take images of the cells using one or more of visible light, UV light and fluorescent light. Alternatively, the cells may be imaged after about 25 to about 200 hours of contact with each treatment. The cells may be imaged once or multiple times, prior to or during contact with each treatment. Of course, any method for determining the viability of the cells may be used to assess the efficacy of each treatment in vitro.

[026] The output of the assay is a series of dose-response curves for tumor cell survivals under the pressure of a single or combination of drugs, with multiple dose settings each (e.g., ten dose settings). To better quantify the assay results, a scoring algorithm may be used that accommodates a dose-response curve. Specifically, the chemoresponse data are applied to an algorithm to quantify the chemoresponse assay results by determining an adjusted area under curve (aAUC) (see US Application No. 12/252,073, which is hereby incorporated by reference in its entirety).

[027] For example, in certain embodiments, the invention quantifies and/or compares the *in vitro* sensitivity/resistance of cells to drugs having varying mechanisms of action, and thus, in some cases, different dose-response curve shapes. In these embodiments, the invention compares the sensitivity of the patient's cultured cells to a plurality of agents that show some effect on the patient's cells in vitro (e.g., all score sensitive to some degree), so that the most effective agent may be selected for therapy. In such embodiments, an aAUC is calculated to take into account the shape of a dose response curve for any particular drug or drug class. The aAUC takes into account changes in cytotoxicity between dose points along a dose-response curve, and assigns weights relative to the degree of changes in

cytotoxicity between dose points. For example, changes in cytotoxicity between dose points along a dose-response curve may be quantified by a local slope, and the local slopes weighted along the dose-response curve to emphasize cytotoxicity.

[028] For example, aAUC may be calculated as follows.

[029] Step 1: Calculate Cytotoxity Index (CI) for each dose, where CI = $Mean_{drug}$ / $Mean_{control}$.

[030] Step 2: Calculate local slope (S_d) at each dose point, for example, as $S_d = (CI_d - CI_{d-1})$ / Unit of Dose, or $S_d = (CI_{d-1} - CI_d)$ / Unit of Dose.

[031] Step 3: Calculate a slope weight at each dose point, e.g., $W_d = 1 - S_d$.

[032] Step 4: Compute aAUC, where aAUC = $\Sigma W_d CI_d$, and where, d = 1, 2, ..., 10; aAUC ~ (0, 10); And at d = 1, then $CI_{d-1} = 1$. Equation 4 is the summary metric of a dose response curve and may used for subsequent regression over reference outcomes.

[033] Usually, the dose-response curves vary dramatically around middle doses, not in lower or higher dose ranges. Thus, the algorithm in some embodiments need only determine the aAUC for a middle dose range, such as for example (where from 8 to 12 doses are experimentally determined, e.g., about 10 doses), the middle 4, 5, 6, or 8 doses are used to calculate aAUC. In this manner, a truncated dose-response curve might be more informative in outcome prediction by eliminating background noise.

[034] The numerical aAUC value (e.g., test value) may then be evaluated for its effect on the patient's cells. For example, a plurality of drugs may be tested, and aAUC determined as above for each, to determine whether the patient's cells have a sensitive response, intermediate response, or resistant response to each drug.

[035] In some embodiments, the agents are designated as, for example, sensitive, or resistant, or intermediate, by comparing the aAUC test value to one or more cut-off values for the particular drug (e.g., representing sensitive, resistant, and/or intermediate aAUC scores for that drug). The cut-off values for any particular drug may be set or determined in a variety of ways, for example, by determining the distribution of a clinical outcome within a range of corresponding aAUC reference scores. That is, a number of patient tumor specimens are tested for chemosenstivity/resistance to a particular drug prior to treatment, and aAUC quantified for each specimen. Then after clinical treatment with that drug, aAUC values that correspond to a clinical response (e.g., sensitive) and the absence of significant clinical response (e.g., resistant) are determined. Cut-off values may alternatively be determined from population response rates. For example, where a patient population is

known to have a response rate of 30% for the tested drug, the cut-off values may be determined by assigning the top 30% of aAUC scores for that drug as sensitive. Further still, cut-off values may be determined by other statistical measures.

[036] In other embodiments, the aAUC scores may be adjusted for drug or drug class. For example, aAUC values for dose response curves may be regressed over a reference scoring algorithm adjusted for test drugs. The reference scoring algorithm may provide a categorical outcome, for example, sensitive (s), intermediate sensitive (i) and resistant (r), as already described. Logistic regression may be used to incorporate the different information, *i.e.*, three outcome categories, into the scoring algorithm. However, regression can be extended to other forms, such as linear or generalized linear regression, depending on reference outcomes. The regression model may be fitted as the following: Logit (Pref) = α + β (aAUC) + γ (drugs), where γ is a covariate vector and the vector can be extended to clinical and genomic features. The score may be calculated as Score = β (aAUC) + γ (drugs). Since the score is a continuous variable, results may be classified into clinically relevant categories, *i.e.*, sensitive (S), intermediate sensitive (I), and resistant (R), based on the distribution of a reference scoring category or maximized sensitivity and specificity relative to the reference.

[037] Generally, where the tumor is determined to be ER-, and determined to be responsive to the taxane in vitro, the tumor is scored (e.g., identified) as being sensitive to taxane therapy (e.g., paclitaxel or docetaxel). Alternatively, the tumor is determined to be ER+, and/or determined to be non-responsive to the taxane in vitro, and thereby scored (e.g., identified) as being resistant to taxane therapy. The invention thereby provides an accurate test for identifying taxane-responsive breast tumors with high sensitivity and/or high specificity.

Apoptosis markers and gene expression signatures

[038] In some embodiments, the malignant cells cultured as above, are tested for the presence of one or more markers of apoptosis, after contact with paclitaxel, docetaxel, or similar agent. The one or more markers include the presence or absence of increased cleaved caspase-3 or cleaved PARP. The presence or absence of increased expression of these markers may be determined by immunoassay (e.g., by Western blot on cell lysates, or In Cell Western) using standard protocols. The presence of such markers adds confidence for a prediction of taxane sensitivity, especially for ER- tumor specimens, and/or specimens deemed sensitive to taxane therapy in the in vitro chemoresponse assay.

[039] Alternatively, or in addition, the method may comprise preparing a gene expression or proteomic profile for a tumor specimen, and determining the presence or absence of a gene expression or proteomic signature indicative of response to taxane therapy.

[040] A gene expression profile is determined for a tumor tissue or cell sample, such as a tumor sample removed from the patient by surgery or biopsy. The tumor sample may be "fresh," in that it was removed from the patent within about five days of processing, and remains suitable or amenable to culture. In some embodiments, the tumor sample is not "fresh," in that the sample is not suitable or amenable to culture. Tumor samples are generally not fresh after from 3 to 7 days (e.g., about five days) of removal from the patient. The sample may be frozen after removal from the patient, and preserved for later RNA isolation. The sample for RNA isolation may be a formalin-fixed paraffin-embedded (FFPE) tissue.

[041] Alternatively, gene expression or proteomic profiles may be prepared from malignant cell cultures (as described above for the chemoresponse assay), before and/or after contact with a therapeutic agent (e.g., paclitaxel).

[042] In preparing the gene expression profile, RNA is extracted from the tumor tissue or cultured cells by any known method. For example, RNA may be purified from cells using a variety of standard procedures as described, for example, in RNA Methodologies, A laboratory guide for isolation and characterization, 2nd edition, 1998, Robert E. Farrell, Jr., Ed., Academic Press. In addition, there are various products commercially available for RNA isolation which may be used. Total RNA or polyA+ RNA may be used for preparing gene expression profiles in accordance with the invention.

[043] The gene expression profile is then generated for the samples using any of various techniques known in the art, and described in detail elsewhere herein. Such methods generally include, without limitation, hybridization-based assays, such as microarray analysis and similar formats (e.g., Whole Genome DASL™ Assay, Illumina, Inc.), polymerase-based assays, such as RT-PCR (e.g., Taqman™), flap-endonuclease-based assays (e.g., Invader™), as well as direct mRNA capture with branched DNA (QuantiGene™) or Hybrid Capture™ (Digene). Gene expression assays, including internal normalization controls and controls for non-differentially expressed genes are known.

[044] The gene or proteomic expression profile contains gene expression levels for a plurality of genes whose expression levels are predictive or indicative of the tumor's response to taxane therapy. Such genes include three, four, five, six, or more (e.g., all) of

AIF1, BAD, BCL2, BID, BIRC5, CASP1, CASP2, CASP6, CASP7, COX4I1, CYCS, PARP12, and PARP4. See Table 1. These genes, which are involved in apoptotic pathways, are differentially expressed between ER+ and ER- breast tumors. Gene or proteomic profiles in some embodiments may contain levels of expression for from 2 to about 1000 genes, or in some embodiments, no more than about 500, about 200, or about 100 genes. Microarray or bead formats for determining large gene expression profiles are well known.

[045] As used herein, "differentially expressed" means that the level or abundance of an RNA transcript (or abundance of an RNA population sharing a common target (or probe-hybridizing) sequence, such as a group of splice variant RNAs) is significantly higher or lower in a drug-sensitive sample as compared to a reference level (e.g., a drug resistant sample). For example, the level of the RNA or RNA population may be higher or lower than a reference level. The reference level may be the level of the same RNA or RNA population in a control sample or control population (e.g., a Mean level for a drug-resistant sample), or may represent a cut-off or threshold level or fold change level for a sensitive or resistant designation.

[046] This expression profile is evaluated for the presence of one or more gene signatures indicative of the tumor's sensitivity and/or resistance to chemotherapeutic agents, and particularly paclitaxel. The gene expression signature comprises the gene expression levels (or numeric values derived therefrom) indicative of a drug-sensitive and/or drug-resistant cell, so as to enable a classification of the tumor's profile as sensitive or resistant. The gene expression signature(s) may be in a format consistent with any nucleic acid detection format, such as those described above, and will generally be comparable to the format used for profiling patient samples. For example, the gene expression signature and patient profiles may both be prepared by nucleic acid hybridization method, and with the same hybridization platform and controls so as to facilitate comparisons. The gene expression signatures may further embody any number of statistical measures to distinguish drug-sensitive and/or drug-resistant levels, including Mean and Median expression levels, and/or cut-off or threshold gene expression or fold change values. For example, the gene expression signature may be manifested as a fold change or Mean or Median fold change between sensitive and resistant cell populations. Such signatures may be prepared from the data sets disclosed herein or independent gene expression data sets.

[047] Once the gene expression profile for patient samples are prepared, the profile is evaluated for the presence of one or more of the gene signatures, by scoring or classifying the patient profile against the gene signature. Various classification schemes are known for classifying samples between two or more classes or groups, and these include, without

limitation: Principal Components Analysis, Naïve Bayes, Support Vector Machines, Nearest Neighbors, Decision Trees, Logistic, Artificial Neural Networks, and Rule-based schemes. In addition, the predictions from multiple models can be combined to generate an overall prediction.

[048] Thus, a classification algorithm or "class predictor" may be constructed to classify samples. The process for preparing a suitable class predictor is reviewed in R. Simon, Diagnostic and prognostic prediction using gene expression profiles in high-dimensional microarray data, *British Journal of Cancer* (2003) 89, 1599-1604, which review is hereby incorporated by reference in its entirety.

[049] Generally, the gene expression profiles for patient specimens are scored or classified as drug-sensitive signatures or drug-resistant signatures, including with stratified or continuous intermediate classifications or scores reflective of drug sensitivity. As discussed, such signatures may be assembled from gene expression data disclosed herein, or prepared from independent data sets. The signatures may be stored in a database and correlated to patient tumor gene expression profiles in response to user inputs.

[050] After comparing the patient's gene expression profile to the drug-sensitive and/or drug-resistant signature, the sample is classified as, or for example, given a probability of being, a drug-sensitive profile or a drug-resistant profile. The classification may be determined computationally based upon known methods as described above. The result of the computation may be displayed on a computer screen or presented in a tangible form, for example, as a probability (e.g., from 0 to 100%) of the patient responding to a given treatment. The report will aid a physician in selecting a course of treatment for the cancer patient. For example, in certain embodiments of the invention, the patient's gene expression profile will be determined to be a drug-sensitive profile on the basis of a probability, and the patient will be subsequently treated with that drug or combination. In other embodiments, the patient's profile will be determined to be a drug-resistant profile, thereby allowing the physician to exclude that candidate treatment for the patient, thereby sparing the patient the unnecessary toxicity.

[051] In various embodiments, the method according to this aspect of the invention distinguishes a drug-sensitive tumor from a drug-resistant tumor with at least about 60%, 75%, 80%, 85%, 90% or greater accuracy.

[052] The methods of the invention aid the prediction of an outcome of treatment. That is, the gene expression signatures are each predictive of an outcome upon treatment with a candidate agent or combination. The outcome may be quantified in a number of ways. For

example, the outcome may be an objective response, a clinical response, or a pathological response to a candidate treatment. The outcome may be determined based upon the techniques for evaluating response to treatment of solid tumors as described in Therasse et al., New Guidelines to Evaluate the Response to Treatment in Solid Tumors, *J. of the National Cancer Institute* 92(3):205-207 (2000), which is hereby incorporated by reference in its entirety. For example, the outcome may be survival (including overall survival or the duration of survival), progression-free interval, or survival after recurrence. The timing or duration of such events may be determined from about the time of diagnosis or from about the time treatment (e.g., chemotherapy) is initiated. Alternatively, the outcome may be based upon a reduction in tumor size, tumor volume, or tumor metabolism, or based upon overall tumor burden, or based upon levels of serum markers especially where elevated in the disease state. The outcome in some embodiments may be characterized as a complete response, a partial response, stable disease, and progressive disease, as these terms are understood in the art.

[053] In certain embodiments, the gene signature is indicative of a pathological complete response upon treatment with paclitaxel or docetaxel, or in combination with other agents (as already described). A pathological complete response, e.g., as determined by a pathologist following examination of tissue (e.g., breast or nodes) removed at the time of surgery, generally refers to an absence of histological evidence of invasive tumor cells in the surgical specimen.

EXAMPLES

Methods

[054] *Chemoresponse assay*: The ChemoFx live cell chemoresponse assay was performed on 25 breast cancer cell lines (10 ER+ and 15 ER-) and 12 primary cultures from 12 human breast cancer specimens. These cells were treated with a 10-dose range of paclitaxel for 72 hours before DAPI-nuclear staining and counting. AUC (Area Under Curve) values were calculated and additional statistical analysis was performed on the resulting dose-response curves.

[055] *RNA Microarray data analysis*: The microarray data for one hundred thirty-three patients with stage I-III breast cancer (Reference: Hess, et. al, Pharmacogenomic Predictor of Sensitivity to Preoperative Chemotherapy With Paclitaxel and 5-Fluorouracil, Doxorubicin, and Cyclophosphamide in Breast Cancer, *Journal of Clinical Oncology*, 24 (26), 2006.) were

downloaded from the public available website http://bioinformatics.mdanderson.org/pubdata.html.

[056] Among these 133 patients, 51 are ER negative, 82 are ER positive. Data were log2-transformed. Non-specific gene filtering was applied to these data sets by software package R and Bioconductor. Suppose x denotes the expression values of probe i, then probes which do not satisfy the following two conditions have been filtered out: 1) IQR(x)<0.5; 2) median(x) <log2(100).

[057] After non-specific filtering, gene matching was conducted, i.e., by matching gene symbol ID with Affy Matrix ID using the hgu133a database. When one symbol ID was matched with multiple affymetrix IDs, the one with the maximum IQR was selected.

[058] Finally, differentially expressed genes in ER+ vs ER- were analyzed by R package LIMMA (Linear Models for Microarray Data). Genes with Q-value less than 0.05 are considered significantly different in expression levels between ER+ and ER- populations.

[059] The fold change is calculated by the following formula: Let Gi represent the raw expression of gene G in sample i, suppose the first m samples are ER positive, and the last n samples are ER negative, then the fold change for gene G is:

$$\frac{\sum_{i=1}^{i=m}\log 2(G_i)}{m} - \frac{\sum_{i=m+1}^{i=m+n}\log 2(G_i)}{n}$$

[060] *Western blot analysis*: 2 of the 25 breast cancer cell lines, T47D (ER+) and SKBR3 (ER-), were treated with paclitaxel at two different dosages (0.0063μM and 0.05μM) for 30h, lysed in RIPA buffer, and total protein concentrations were measured with Bradford assay. Total of 60μg proteins from each of the resulting cell lysates were loaded onto SDS-PAGE gel, followed by electrophoresis and transferring to nitrocellulose membranes, and blotted with anti-cleaved caspase-3 (Cell Signaling #9661), and anti-cleaved PARP antibody (Cell Signaling #9546) to evaluate apoptosis pathway, and with anti-beta-actin as a normal control.

[061] To confirm these results, a separate experiment was conducted with six of the 25 immortalized breast cancer cell lunes (BT474, AU565, SKBR3, HCC1500, HCC1569 and HCC38). The cells were cultured in 10% RPMI for 24 hours before they were treated, or not treated, with paclitaxel at two different dosages (0.012 μM and 0.05 μM) for 40 hours and lysed in RIPA buffer (Cell Signaling Technology Inc., Danvers, MA). A total of 100 μg proteins from each of the resulting cell lysates was loaded onto SDS-PAGE gels, followed by

electrophoresis and transfer to nitrocellulose membranes, and blotted with anti-cleaved caspase-3 (Cell Signaling #9661), and anti-cleaved PARP antibody (Cell Signaling #9546) to evaluate apoptosis pathway, and with anti-beta-actin as a normal control.

[062] *In Cell Western blot analysis*: In-cell Western analysis was performed on the same 6 immortalized breast cancer cell lines (BT474, AU565, SKBR3, HCC1500, HCC1569, and HCC38) and cells from the same 12 primary cultures established from 12 breast cancer tissue specimens were plated onto 384-well plates at the density of 2,000 cells per well in 10% RPMI for cell lines and in 10% MEGM for primary cultures and cultured for 24 hours; they then were treated, or not treated, with paclitaxel at two different dosages (0.012μM and 0.05μM) for 40 hours. Cells were fixed by 4% formaldehyde at room temperature for 20 minutes, permeabilized with 0.25% Triton in PBS, and washed with PBS three times before the blocking step for 1 hour at room temperature with 50ul of blocking buffer (1% BSA in PBST) in each well. The blocking buffer was then removed and replaced with new blocking buffer in the negative control wells; rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology Inc.) diluted in blocking buffer at the ratio of 1:200 was added to the experimental wells and incubated overnight at 4° C with gentle agitation. The wells were then washed with PBS three times with moderate agitation.

[063] A secondary antibody solution in blocking buffer with IRdye800 labeled goat anti-rabbit 2nd antibody was added to negative control wells, and a secondary antibody solution with IRdye800 labeled goat anti-rabbit 2nd antibody (800-channel) and DNA dyes (700-channel) was added to the experimental wells (LI-COR, Lincoln, NE, USA). Plates were wrapped in foil and incubated at room temperature for 1 hour. The wells were then washed with PBS three times, blotted with paper towels and then imaged on the Odyssey system and analyzed with Odyssey software (LI-COR). Integrated intensity ratios were calculated for each cell line or primary culture by subtracting the background intensity from the negative control intensity for the channel and then dividing the intensity at the 800-channel by the intensity at the 700-channel (800/700).

Results

[064] Results from ChemoFx® on the 25 immortalized breast cancer cell lines (10 ER+ and 15 ER–) are shown in **Figure 1a and 1c**. Notably, none of the 10 ER+ breast cell lines was categorized as R (responsive) to paclitaxel, with 8 NR (non-responsive) and 2 IR (intermediate responsive). In contrast, only 4 of the 15 ER– breast cancer cell lines were categorized as NR; 8 were IR, and 3 were R. See **Figure 1b.** Based on Wilcoxon rank-sum

test, this suggests that the ER– breast cancer cells are more likely to respond to paclitaxel than are ER+ cells (median RI score 5.81 vs 5.00, $p<0.001$).

[065] Of the 12 breast cancer primary cultures (A to L) established from 12 breast cancer patients' tumor specimens, 8 were revealed as ER+ and 4 as ER–, based on Western blot analysis. See **Figure 2c**. Results from ChemoFx® on these breast cancer cells showed that none of the 8 ER+ breast cancer cells was categorized as R to paclitaxel, with 6 NR and 2 IR. In contrast, 2 of the 4 ER– breast cancer cell lines were categorized as R; 2 were IR. Based on Wilcoxon rank-sum test, this suggests that the ER– cells were more likely to respond to paclitaxel than ER+ cells (median RI score: 5.71 vs 5.06, $p=0.12$) See **Figures 2a** and **2b**.

[066] Microarray analysis revealed differential expressions of genes implicated in the apoptosis pathway ($q< 0.05$) in ER+ and ER- breast cancers. See Table 1. The polynucleotide sequences of these genes and associated primers are known, and are hereby incorporated by reference.

[067] Western blot analysis showed that paclitaxel induced cleaved caspase-3 and cleaved PARP expressions, both of which are indicators of activation of apoptosis, in SKBR3 cells (ER-), but not in T47D cells (ER+) (not shown).

[068] Furthermore, upon paclitaxel treatment, cleaved caspase-3 and cleaved PARP were detected in AU565, SKBR3, and HCC38, all of which are responsive cell lines to paclitaxel according to ChemoFx®. These expressions were not detected in the paclitaxel-non-responsive cell lines, which included BT474, HCC1500, and HCC1569 (See **Figure 3a**). In-cell Western analysis confirmed that upon paclitaxel treatment, cleaved caspase-3 levels increased dramatically in AU565, SKBR3, and HCC38, all of which were ER– breast cancer cell lines that were responsive to paclitaxel according to ChemoFx®; this increase in cleaved caspase-3 levels did not occur in the paclitaxel-non-responsive cell lines including BT474, HCC1500, and HCC1569 (See **Figure 3b**). All of the responsive cell lines tested for Western blot analysis and in-cell Western analysis were ER– breast cancer cell lines, none of the ER+ breast cancer cell lines were responsive to paclitaxel according to ChemoFx®. Among these non-responsive cell lines tested, BT474 and HCC1500 were ER+ and HCC1569 was ER– (See **Figure 1c**).

[069] Of the 12 breast cancer primary cultures (A to L), in-cell Western analysis demonstrated that upon paclitaxel treatment, cleaved caspase-3 levels increased dramatically in C, F, K and L (see **Figure 4**), all of which were either breast cancer cells that were responsive to paclitaxel (C and F) or breast cancer cells that were intermediate

responsive to paclitaxel (K and L), according to ChemoFx® (see **Figure 2**). This increase in cleaved caspase-3 levels did not occur in the paclitaxel-non-responsive breast cancer cells including A, B, D, E, G, H, I and J (see **Figure 5**). Of these 12 breast cancer primary cultures (A to L), 8 were revealed as ER+ (A, B, E, H, I J, K and L, among which ER expression in B, I and K were weaker) and 4 as ER– (C, D, F and G), based on Western blot analysis (see **Figure 2**).

Conclusions

[070] ER status predicts in part, the response of breast cancer cells to paclitaxel, with additional predictive value provided by the ChemoFx assay. ER-negative breast cancer cells are more likely to be responsive, which is consistent with established clinical findings. The chemoresponse assay also distinguishes between NR/IR and R to paclitaxel within the ER- population. Results from RNA microarray and Western blot analyses show differences in gene expression in the apoptosis pathway, and in activation of apoptosis pathway, namely changes in expressions of cleaved PARP and cleaved caspase-3 in response to paclitaxel. Such markers may explain differences in the responsiveness of ER+ and ER- breast cancers to paclitaxel, and provide further levels of predictive ability in place of or in addition to in vitro chemoresponse testing. Specifically, this suggests a role of cleaved PARP and cleaved caspase-3 as biomarkers, in addition to ER for example, for prediction of paclitaxel responsiveness in breast cancer.

[071] All references cited herein, including patents and published patent applications, as well as all non-patent literature, are each hereby incorporated by reference in their entireties.

Table 1

Symbol	Probe_ID	Mean log_ER–	Mean log_ER+	q-val
A1F1	215051_x_at	10.18	9.91	4.35E-02
BAD	1438_a1	6.67	6.97	5.49E-04
BCL2	203685_at	6.94	6.16	7.17E-11
BID	211725_s_at	8.18	7.71	1.74E-05
BIRC5	202095_s_at	8.89	8.56	4.63E-02
CASP1	21136_x_at	8.96	8.62	1.51E-03
CASP2	33768_at	7.57	7.39	2.41E01
CASP6	211464_x_at	6.53	6.99	1/20E-02
COX411	202698_x_at	11.83	11.57	6.81E-03
CYCS	206974_at	10.85	10.49	3.06E-03
PARP12	212345_s_at	8.73	8.41	1.44E-02
PARP4	216822_x_at	7.98	7.72	3.89E-03

CLAIMS:

1. A method for determining the sensitivity and/or resistance of a breast tumor to taxane therapy, comprising:

determining an estrogen receptor (ER) status for the tumor;

determining an *in vitro* chemosensitivity of malignant cells derived from the tumor to a taxane; and/or

determining the presence, absence, or level of one or more markers indicative of apoptosis in response to the taxane.

2. The method of claim 1, wherein the taxane is paclitaxel or docetaxel.

3. The method of claim 1 or 2, wherein the tumor is determined to be ER-.

4. The method of any one of claims 1 to 3, wherein determining *in vitro* chemosensitivity comprises preparing a cell culture from the tumor that is enriched for malignant cells, and testing the malignant cells for an *in vitro* response to the taxane.

5. The method of claim 4, wherein the cell culture is prepared by a process comprising:

disaggregating a specimen of the breast tumor to create a plurality of tissue explants;

allowing the plurality of tissue explants to form a monolayer, and transferring the cells of the monolayer to a plurality of test sites for chemosensitivity testing.

6. The method of claim 5, where the tumor is determined to be ER-, and determined to be responsive to the taxane *in vitro*, and thereby scored as being sensitive to the taxane.

7. The method of claim 5, where the tumor is determined to be ER+, and/or determined to be non-responsive to the taxane *in vitro*, and thereby scored as being resistant to the taxane.

8. The method of any one of claims 1 to 5, wherein the one or more markers indicative of apoptosis is a level of expression of at least three genes or proteins selected from AIF1, BAD, BCL2, BID, BIRC5, CASP1, CASP6, CASP7, COX4I1, CYCS, PARP12, and PARP4.

9. The method of any one of claims 1 to 5, wherein the one or more markers indicative of apoptosis is a protein marker for activated apoptosis.

10. The method of claim 9, wherein the marker is caspase-3 cleavage or cleaved PARP.

11. A method for determining the sensitivity and/or resistance of an ER- breast tumor to a taxane, comprising:

determining an in vitro chemosensitivity of the tumor to the taxane; and/or

determining the presence, absence, or level of one or more markers indicative of apoptosis or response to the taxane.

12. The method of claim 11, wherein the taxane is paclitaxel or docetaxel.

13. The method of claim 11 or 12, wherein determining *in vitro* chemosensitivity comprises preparing a cell culture from the tumor that is enriched for malignant cells, and testing the malignant cells for an *in vitro* response to the taxane.

14. The method of claim 13, wherein the cell culture is prepared by a process comprising:

disaggregating a specimen of the breast tumor to create a plurality of tissue explants;

allowing the plurality of tissue explants to form a monolayer, and transferring the cells of the monolayer to a plurality of test sites for chemosensitivity testing.

15. The method of any one of claims 11 to 14, wherein one or more markers indicative of apoptosis is a level of expression of at least one gene selected from AIF1, BAD, BCL2, BID, BIRC5, CASP1, CASP6, CASP7, COX4I1, CYCS, PARP12, and PARP4.

16. The method of any one of claims 11 to 14, wherein the one or more markers indicative of apoptosis comprises a protein marker for activated apoptosis.

17. The method of claim 16, wherein the marker is caspase-3 cleavage or cleaved PARP.

18. A method for identifying a breast tumor that is response to taxane therapy, comprising:

determining an ER status of the tumor, wherein an ER+ status is predictive that the tumor will be non-responsive or intermediate responsive to taxane therapy;

where the tumor is ER-, determining an in vitro chemosensitivity of malignant cells derived from the tumor to a taxane, and/or determining the presence, absence, or level of one or more markers indicative of apoptosis in response to the taxane, to thereby identify a breast tumor that is responsive to taxane therapy.

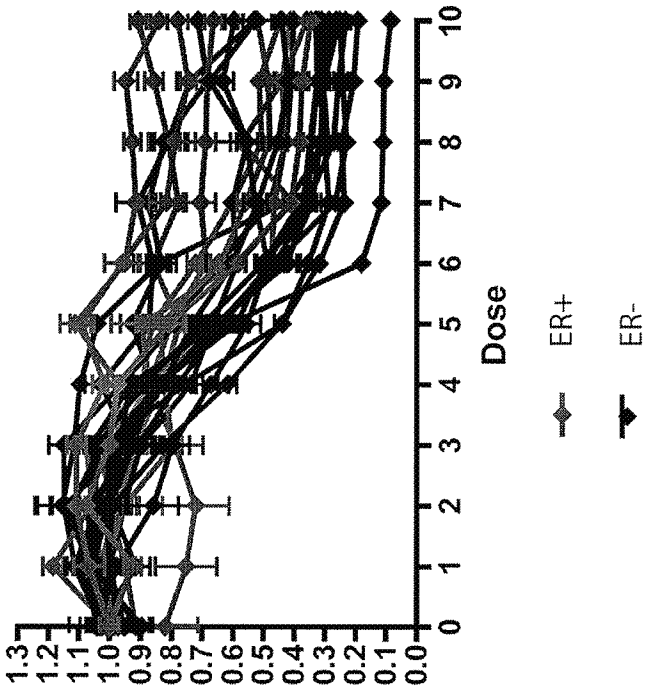


Figure 1a

	ER+ (10)	ER- (15)
Responsive (R)	0	3
Intermediate Responsive (IR)	2	8
Non Responsive (NR)	8	4

Figure 1b

Figure 1c

Cell line	ER status	ChemoFx response
AU565	neg	R
BT20	neg	IR
BT474	pos	NR
BT483	pos	NR
BT549	neg	IR
HCC1143	neg	IR
HCC1187	neg	IR
HCC1428	pos	NR
HCC1500	pos	NR
HCC1569	neg	NR
HCC1937	neg	IR
HCC1954	neg	IR
HCC202	neg	NR
HCC38	neg	R
MCF7	pos	IR
MDAMB231	neg	IR
MDAMB361	pos	NR
MDAMB415	pos	NR
MDAMB436	neg	NR
MDAMB453	neg	NR
MDAMB468	neg	IR
SKBR3	neg	R
T47D	pos	IR
UACC812	pos	NR
ZR751	pos	NR

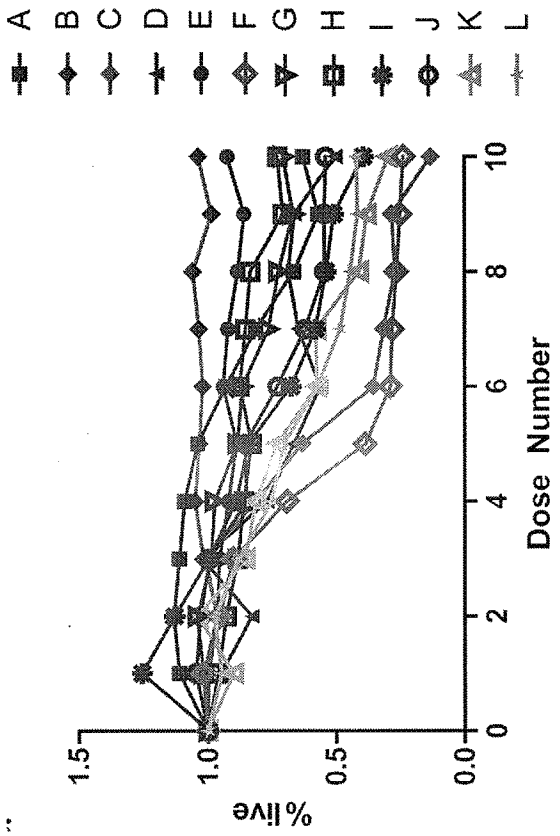


Figure 2a

	ER+ (8)	ER- (4)
Responsive (R)	0	2
Intermediate Responsive (IR)	2	0
Non Responsive (NR)	6	2

Figure 2b

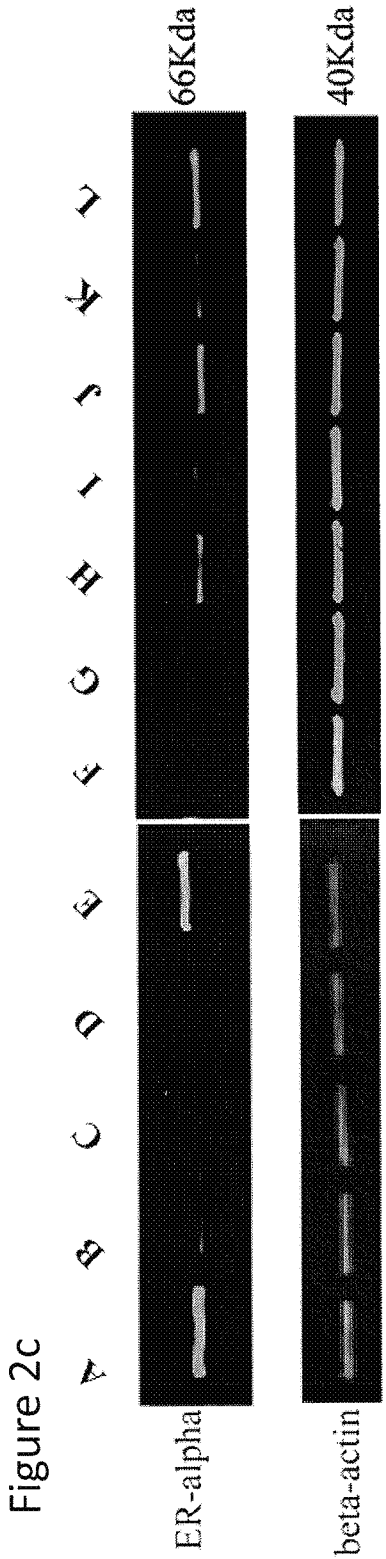
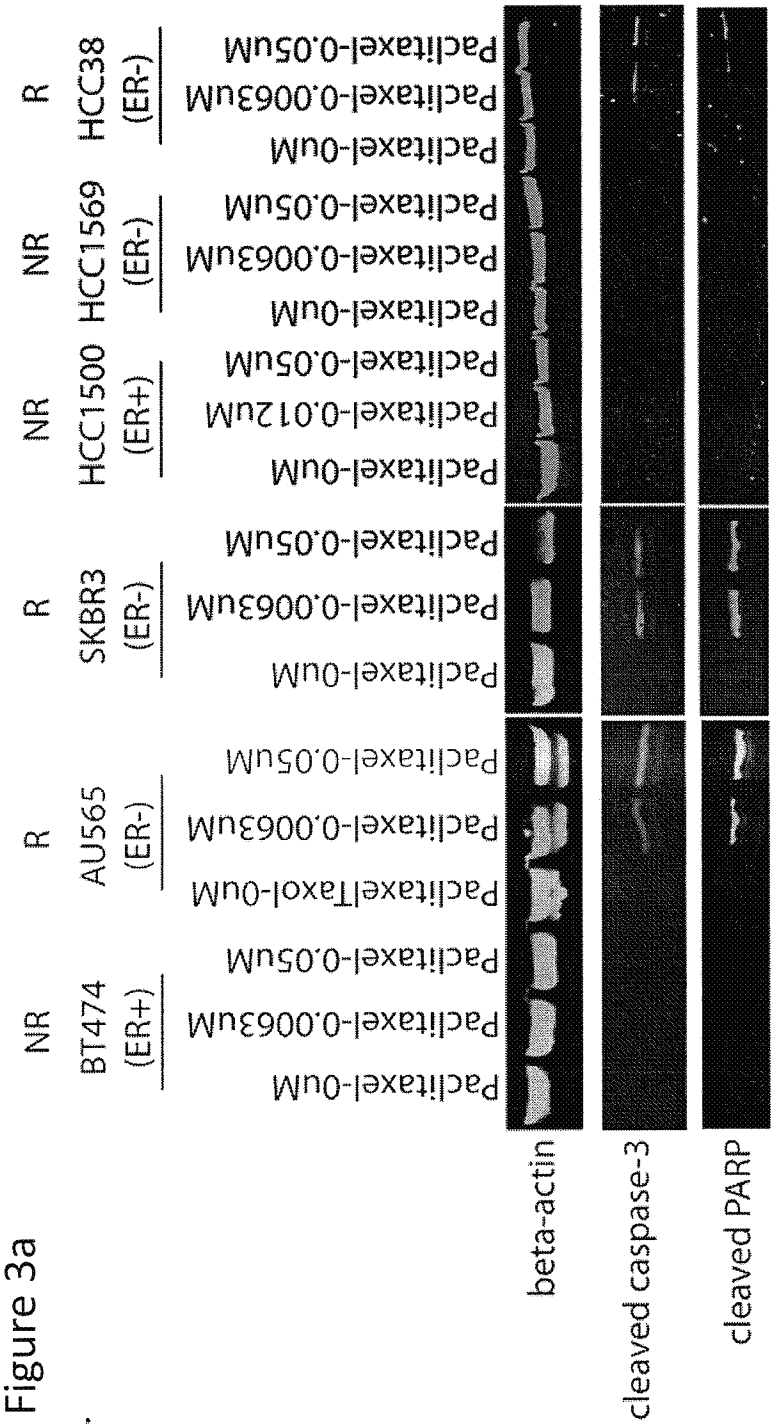


Figure 2c

Figure 3a


NR
BT474 (ER+)
Paclitaxel-0uM
Paclitaxel-0.0063uM
Paclitaxel-0.05uM
R
AU565 (ER-)
PaclitaxelTaxol-0uM
Paclitaxel-0.0063uM
Paclitaxel-0.05uM
R
SKBR3 (ER-)
Paclitaxel-0uM
Paclitaxel-0.0063uM
Paclitaxel-0.05uM
NR
HCC1500 (ER+)
Paclitaxel-0uM
Paclitaxel-0.012uM
Paclitaxel-0.05uM
NR
HCC1569 (ER-)
Paclitaxel-0uM
Paclitaxel-0.0063uM
Paclitaxel-0.05uM
R
HCC38 (ER-)
Paclitaxel-0uM
Paclitaxel-0.0063uM
Paclitaxel-0.05uM
beta-actin
cleaved caspase-3
cleaved PARP

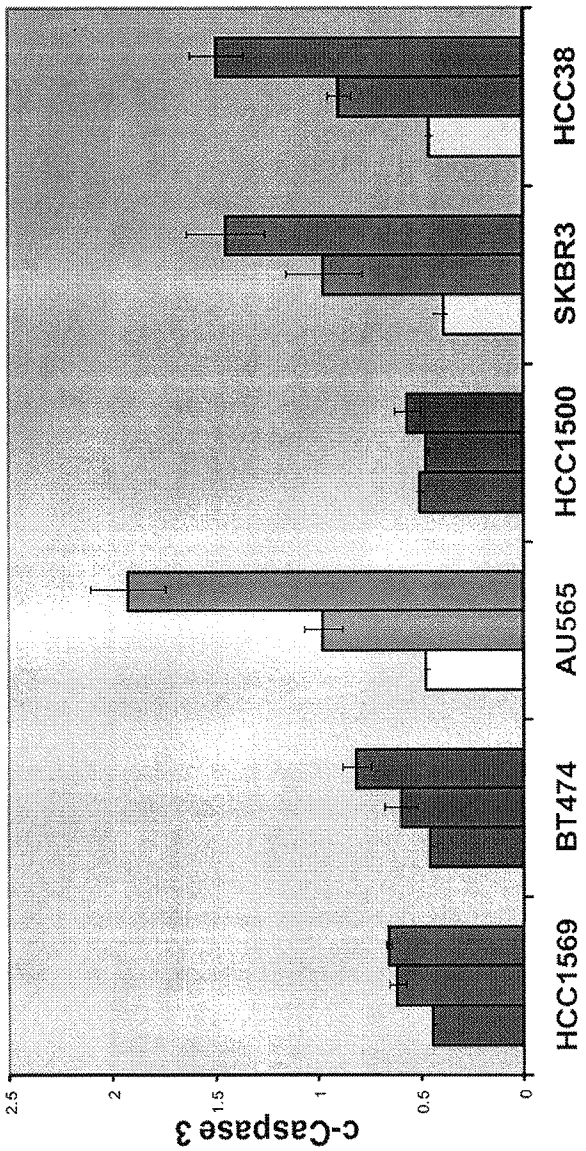
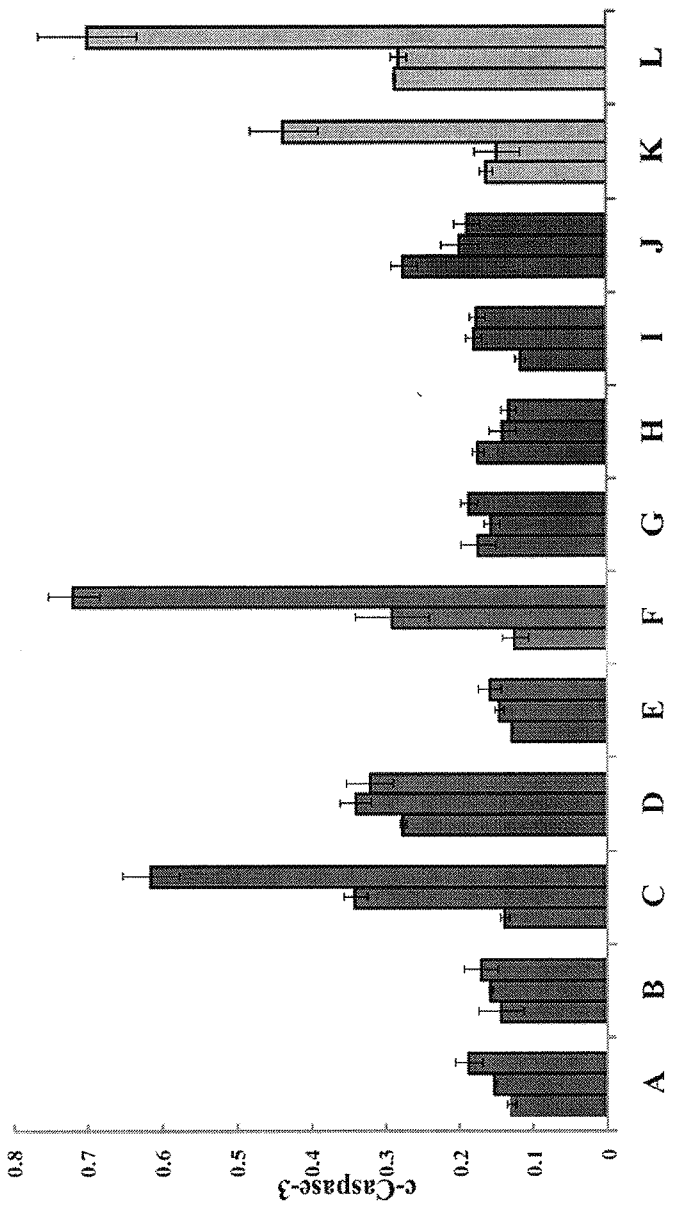

c-Caspase 3
2.5
2
1.5
1
0.5
0
HCC1569
BT474
AU565
HCC1500
SKBR3
HCC38

Figure 3b

Figure 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38641

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/574
USPC - 435/7.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - G01N 33/574
USPC - 435/7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - G01N 33/574; C12Q 1/68; A61K 31/337, 31/7048, 31/353, 31/12
USPC - 435/7.23, 6; 514/449, 27, 456, 680

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (PGPB, USPT, EPAB, JPAB); Google Scholar
Search Terms Used: (TAXANE? "ESTROGEN RECEPTOR STATUS" NEGATIVE), (TAXANE? "ESTROGEN RECEPTOR STATUS"), (TAXANE? ESTROGEN RECEPTOR), (er status taxane), (estrogen cancer "ER-negative" +(taxane OR paclitaxel OR decetaxel))

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2005/0131057 A1 (Ueno et al.) 16 June 2005 (16.06.2005), the entire document, especially abstract, para[0003], para[0013], para[0016]	1,2,11-13,18 ---------- 3, 14
Y	MAZOUNI et al., Inclusion of taxanes, particularly weekly paclitaxel, in preoperative chemotherapy improves pathologic complete response rate in estrogen receptor-positive breast cancers, Annals Onc. Vol. 18, p. 874-880 (2007)	3
Y	KUSNETSOV et al., Potent in vitro and in vivo anticancer activities of des-methyl, des-amino pateamine A, a synthetic analogue of marine natural product pateamine A, Mol. Cancer Ther., May 2009 Vol. 8, No. 5, p1251-1260.	14

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 2010 (17.07.2010)	10 AUG 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38641

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-10, 15-17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.