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(54) **Title**: THERANOSTIC AND DIAGNOSTIC METHODS USING SPARC AND HSP90

(57) **Abstract**: Provided herein are methods and systems of molecular profiling of diseases, such as cancer. The molecular profiling can be used to provide a diagnosis, prognosis, or theranosis for the disease, such as identifying a candidate treatment. The methods can detect overexpression of SPARC and HSP90. The cancer can be, e.g., a renal cell carcinoma or an interdigitating dendritic cell sarcoma.

THERANOSTIC AND DIAGNOSTIC METHODS USING SPARC AND HSP90

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Patent Application 12/211,765, filed on September 16, 2008; and this application claims the benefit of U.S. Provisional Patent Application 61/314,942, filed on March 17, 2010; all of which applications are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Disease states in patients are typically treated with treatment regimens or therapies that are selected based on clinical based criteria; that is, a treatment therapy or regimen is selected for a patient based on the determination that the patient has been diagnosed with a particular disease (which diagnosis has been made from classical diagnostic assays). Although the molecular mechanisms behind various disease states have been the subject of studies for years, the specific application of a diseased individual's molecular profile in determining treatment regimens and therapies for that individual has been disease specific and not widely pursued.

[0003] Some treatment regimens have been determined using molecular profiling in combination with clinical characterization of a patient such as observations made by a physician (such as a code from the International Classification of Diseases, for example, and the dates such codes were determined), laboratory test results, x-rays, biopsy results, statements made by the patient, and any other medical information typically relied upon by a physician to make a diagnosis in a specific disease. However, using a combination of selection material based on molecular profiling and clinical characterizations (such as the diagnosis of a particular type of cancer) to determine a treatment regimen or therapy presents a risk that an effective treatment regimen may be overlooked for a particular individual since some treatment regimens may work well for different disease states even though they are associated with treating a particular type of disease state.

[0004] Patients with refractory or metastatic cancer are of particular concern for treating physicians. The majority of patients with metastatic or refractory cancer eventually run out of treatment options or may suffer a cancer type with no real treatment options. For example, some patients have very limited options after their tumor has progressed in spite of front line, second line and sometimes third line and beyond) therapies. For these patients, molecular profiling of their cancer may provide the only viable option for prolonging life.

[0005] More particularly, additional targets or specific therapeutic agents can be identified assessment of a comprehensive number of targets or molecular findings examining molecular mechanisms, genes, gene expressed proteins, and/or combinations of such in a patient's tumor. Identifying multiple agents that can treat multiple targets or underlying mechanisms would provide cancer patients with a viable therapeutic alternative on a personalized basis so as to avoid standar therapies, which may simply not work or identify therapies that would not otherwise be considered by the treating physician.

[0006] There remains a need for better theranostic assessment of cancer vicitims, including molecular profiling analysis that identifies one or more individual profiles to provide more informed and effective personalized treatment options, resulting in improved patient care and enhanced treatment outcomes. The present invention provides methods and systems for identifying treatments for these individuals by molecular profiling a sample from the individual. The molecular profiling can include profiling of SPARC and/or HSP90. The profiling can be used for diagnosis, prognosis and theranosis of a malignancy.

SUMMARY OF THE INVENTION

[0007] The present invention provides methods and system for molecular profiling, using the results from molecular profiling to identify treatments for individuals. In some embodiments, the treatments were not identified initially as a treatment for the disease.

[0008] In an aspect, the present invention provides a method of selecting a candidate treatment for a malignancy in a subject comprising: obtaining a sample of the malignancy; detecting a level of SPARC and HSP90 in the sample; and selecting a treatment associated with SPARC and HSP90 if the sample has an elevated level of SPARC and HSP90 as compared to a reference. The reference can be from a non-malignant sample. The reference can be from the subject.

[0009] The level of SPARC and HSP90 can be detected using a variety of molecular profiling methods, such as one or more of IHC, FISH, PCR, microarray and sequencing. The microarray analysis can be a low density microarray, an expression microarray, a comparative genomic hybridization (CGH) microarray, a single nucleotide polymorphism (SNP) microarray, a proteomic array or an antibody array. In some embodiments, the microarray analysis comprises identifying whether a gene is significantly upregulated or downregulated relative to a reference. The significance can be determined at a p-value, e.g., a p-value of less than or equal to 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001. The p-value can be corrected for multiple comparisons. Such correction can be made using Bonneferoni's correction, a false discovery rate, or a modification of any thereof.

[0010] In some embodiments, the IHC analysis comprises determining whether 30% or more of the sample is +2 or greater in staining intensity. The SPARC and HSP90 can be considered elevated if 30% or more of the sample is +2 or greater in staining intensity for SPARC and HSP90 as determined by the IHC analysis.

[0011] The methods of the invention can be used to identify a prioritized list of candidate treatments. Such prioritization can depend on how the levels are identified. For example, prioritizing may comprise ordering the candidate treatments from higher priority to lower priority according to treatments based on either IHC or FISH analysis of the SPARC and HSP90 and microarray analysis; treatments based on IHC analysis of the SPARC and HSP90 but not microarray analysis; and treatments based on microarray analysis of the SPARC and HSP90 but not IHC analysis.

[0012] The candidate treatments identified by the invention can include one or more therapeutic agent. In some embodiments, the one or more therapeutic agent comprises one or more mitotic inhibitor. The one or more mitotic inhibitor can be a taxane, a vinca alkaloid, or a combination thereof. The taxane can be paclitaxel, nab-paclitaxel, paclitaxel bound to albumin, or docetaxel. In embodiments, the vinca alkaloid comprises vincristine, vinblastine, vindesine or vinorelbine. The one or more therapeutic agent can further comprise one or more HSP90 inhibitor, including without limitation geldanamycin, 17-*N*-Allylamino-17-demethoxygeldanamycin (17-AAG), 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), IPI-504 (retaspimycin), BIIB021 (CNF2024), BIIB028, SNX-5422, Ganetespib STA-9090, AUY922, AT13387, cisplatin, herbimycin, radicicol, novobiocin, coumermycin A1, clorobiocin, epigallocatechin gallate (EGCG), taxol, pochonin, derrubone, gedunin, celastrol, or a derivative of any thereof.

[0013] The methods for identifying a candidate treatment can comprise molecular profiling of additional genes and gene products in addition to HSP90 or SPARC. For example, molecular profiling can also be performed for one or more of ABCC1, ABCG2, ACE2, ADA, ADH1C, ADH4, AGT, AR, AREG, ASNS, BCL2, BCRP, BDCA1, beta III tubulin, BIRC5, B-RAF, BRCA1, BRCA2, CA2, caveolin, CD20, CD25, CD33, CD52, CDA,

CDKN2A, CDKN1A, CDKN1B, CDK2, CDW52, CES2, CK 14, CK 17, CK 5/6, c-KIT, c-Met, c-Myc, COX-2, Cyclin D1, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, E-Cadherin, ECGF1, EGFR, EML4-ALK fusion, EPHA2, Epiregulin, ER, ERBR2, ERCC1, ERCC3, EREG, ESR1, FLT1, folate receptor, FOLR1, FOLR2, FSHB, FSHPRH1, FSHR, FYN, GART, GNRH1, GNRHR1, GSTP1, HCK, HDAC1, hENT-1, Her2/Neu, HGF, HIF1A, HIG1, HSPCA, HSP90AA1, IGF-1R, IGFRBP, IGFRBP3, IGFRBP4, IGFRBP5, IL13RA1, IL2RA, KDR, Ki67, KIT, K-RAS, LCK, LTB, Lymphotoxin Beta Receptor, LYN, MET, MGMT, MLH1, MMR, MRP1, MS4A1, MSH2, MSH5, Myc, NFKB1, NFKB2, NFKBIA, ODC1, OGFR, p16, p21, p27, p53, p95, PARP-1, PDGFC, PDGFR, PDGFRA, PDGFRB, PGP, PGR, PI3K, POLA, POLA1, PPARG, PPARGC1, PR, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Survivin, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TS, TXN, TXNRD1, TYMS, VDR, VEGF, VEGFA, VEGFC, VHL, YES1, and ZAP70. Candidate treatments for one or more of these genes can be identified accordingly.

[0014] In some embodiments, the subject has been previously treated with the candidate treatment. In other embodiments, the subject has not previously been treated with one or more candidate therapeutic agents. The malignancy can be a metastatic malignancy. The malignancy can also be a recurrent malignancy. In embodiments, the malignancy is refractory to a prior treatment. The prior treatment can comprise the standard of care for the malignancy. In such cases, the subject methods can be used to identify an alternate to the standard of care.

[0015] The methods can be used to identify a candidate treatment for any sample. For example, the malignancy can be a malignancy of a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, or an adipose tissue. The malignancy can be a carcinoma or sarcoma. The malignancy can be a renal cell carcinoma. The malignancy can be an interdigitating dendritic cell sarcoma. In various embodiments, the malignancy comprises an acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related cancer; AIDS-related lymphoma; anal cancer; appendix cancer; astrocytomas; atypical teratoid/rhabdoid tumor; basal cell carcinoma; bladder cancer; brain stem glioma; brain tumor, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, astrocytomas, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, supratentorial primitive neuroectodermal tumors and pineoblastoma; breast cancer; bronchial tumors; Burkitt lymphoma; cancer of unknown primary site (CUP); carcinoid tumor; carcinoma of unknown primary site; central nervous system atypical teratoid/rhabdoid tumor; central nervous system embryonal tumors; cervical cancer; childhood cancers; chordoma; chronic lymphocytic leukemia; chronic myelogenous leukemia; chronic myeloproliferative disorders; colon cancer; colorectal cancer; craniopharyngioma; cutaneous T-cell lymphoma; endocrine pancreas islet cell tumors; endometrial cancer; ependymoblastoma; ependymoma; esophageal cancer; esthesioneuroblastoma; Ewing sarcoma; extracranial germ cell tumor; extragonadal germ cell tumor; extrahepatic bile duct cancer; gallbladder cancer; gastric (stomach) cancer; gastrointestinal carcinoid tumor; gastrointestinal stromal cell tumor; gastrointestinal stromal tumor (GIST); gestational trophoblastic tumor; glioma; hairy cell leukemia; head and neck cancer; heart cancer; Hodgkin lymphoma; hypopharyngeal cancer; intraocular melanoma; islet cell tumors; Kaposi sarcoma; kidney cancer; Langerhans cell histiocytosis; laryngeal cancer; lip cancer; liver cancer; malignant fibrous histiocytoma bone cancer; medulloblastoma; medulloepithelioma; melanoma; Merkel cell

carcinoma; Merkel cell skin carcinoma; mesothelioma; metastatic squamous neck cancer with occult primary; mouth cancer; multiple endocrine neoplasia syndromes; multiple myeloma; multiple myeloma/plasma cell neoplasm; mycosis fungoides; myelodysplastic syndromes; myeloproliferative neoplasms; nasal cavity cancer; nasopharyngeal cancer; neuroblastoma; Non-Hodgkin lymphoma; nonmelanoma skin cancer; non-small cell lung cancer; oral cancer; oral cavity cancer; oropharyngeal cancer; osteosarcoma; other brain and spinal cord tumors; ovarian cancer; ovarian epithelial cancer; ovarian germ cell tumor; ovarian low malignant potential tumor; pancreatic cancer; papillomatosis; paranasal sinus cancer; parathyroid cancer; pelvic cancer; penile cancer; pharyngeal cancer; pineal parenchymal tumors of intermediate differentiation; pineoblastoma; pituitary tumor; plasma cell neoplasm/multiple myeloma; pleuropulmonary blastoma; primary central nervous system (CNS) lymphoma; primary hepatocellular liver cancer; prostate cancer; rectal cancer; renal cancer; renal cell (kidney) cancer; renal cell cancer; respiratory tract cancer; retinoblastoma; rhabdomyosarcoma; salivary gland cancer; Sézary syndrome; small cell lung cancer; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; squamous neck cancer; stomach (gastric) cancer; supratentorial primitive neuroectodermal tumors; T-cell lymphoma; testicular cancer; throat cancer; thymic carcinoma; thymoma; thyroid cancer; transitional cell cancer; transitional cell cancer of the renal pelvis and ureter; trophoblastic tumor; ureter cancer; urethral cancer; uterine cancer; uterine sarcoma; vaginal cancer; vulvar cancer; Waldenström macroglobulinemia; a Wilm's tumor; or a cancer of unknown primary (CUP).

[0016] The candidate treatment can be used to treat the subject. In an embodiment, progression free survival (PFS) or disease free survival (DFS) for the subject is extended by treatment with the one or more candidate therapeutic. The subject's lifespan can be extended by selection of the candidate treatment.

[0017] In another aspect, the present invention provides a method for detecting an elevated level of SPARC and HSP90 in a biological sample. In one embodiment, the method includes immunohistochemical staining. Immunohistochemistry (IHC) combines several techniques to identify specific tissue components, including the overexpression of various proteins, by using a specific antigen/antibody reaction tagged with a visible label. IHC enables the visualization of the distribution and localization of specific cellular components within a cell or tissue. A typical IHC protocol includes fixing a tissue sample, blocking nonspecific sites in the tissue sample with serum or a blocker protein, incubating the tissue sample with primary antibody, washing the tissue sample buffer, suppressing endogenous peroxidase activity in the tissue sample, washing the tissue sample with buffer, incubating the tissue sample with a HRP-conjugated secondary antibody, washing the tissue sample with buffer, incubating the tissue sample with 3,3' diaminobenzidine (DAB) substrate, and washing the tissue sample with water. Total time for incubating the tissue sample with the various reagents and carrying out all of the steps is typically over 2 hours.

[0018] The methods of the invention improve upon the typical IHC protocol. In some embodiments, the instant method does not use a protein blocking agent to block nonspecific sites in the sample. In addition, in some embodiments, the method of the invention does not perform the step of inhibiting or suppressing endogenous peroxidase activity in the sample after incubating the sample with a primary antibody and before incubating the sample with peroxidase conjugate. Instead, the step of inhibiting or suppressing endogenous peroxidase activity in the sample can be performed prior to incubating the sample with primary antibody. Further, the time frames for exposing various reagents to the sample can be reduced throughout the process

thereby resulting in a method for detecting elevated levels of SPARC and/or HSP90 using IHC which takes between about 1.5 and 1.75 hours to complete.

[0019] The IHC method of detecting an elevated level of SPARC and/or HSP90 in a biological tissue in accordance with the present invention can include: a) providing a slide having a sample contained thereon; b) processing the sample such as deparaffinizing and rehydrating the sample in the event that the sample is a formalin fixed paraffin embedded (FFPE) sample; c) rinsing the sample with buffer and/or other aqueous liquids; d) covering the sample with peroxide, or other suitable oxidative agents for approximately 1 to 60 minutes; e) rinsing the sample with buffer and/or other aqueous liquids; f) covering the sample with a primary SPARC antibody and/or a primary HSP90 antibody diluted in a Tris based diluent, a negative control reagent, or other suitable carrier solution for approximately 5 to 120 minutes; g) rinsing the sample with buffer and/or other suitable aqueous liquids; h) covering the sample with a secondary detection reagent such as an antibody probe reagent, a secondary binding reagent, or a polymer detection reagent; i) rinsing the sample with buffer and/or other aqueous liquids; j) covering the sample with a horseradish peroxidase conjugate, an enzymatic agent, or a catalytic agent; k) rinsing the sample with buffer and/or other aqueous liquids; l) covering the sample with a first volume of at least one of a 3,3'–diaminobenzidine (DAB) containing solution or a chromogen containing solution for 1 to 60 minutes; m) covering the sample with a second volume of at least one of a DAB containing solution or a chromogen containing solution for 1 to 60 minutes; n) rinsing the sample with water and/or other aqueous liquids; o) covering the sample with a counterstain such as a hematoxylin counterstain for approximately 1 to 20 minutes; p) rinsing the sample with buffer and/or other aqueous liquids; q) repeatedly dipping the sample and the slide in water until the sample is clear; r) dehydrating the sample; s) applying a coverslip over the sample contained on the slide; and t) detecting an elevated level of SPARC and/or HSP90 by examining the sample under a microscope and comparing the sample to a control.

[0020] In one exemplary embodiment, the step f) of covering the sample with a primary SPARC antibody and/or primary HSP90 antibody includes covering the sample with a monoclonal antibody and the step of covering the sample with a secondary detection agent includes covering the sample with a monoclonal antibody probe reagent. Covering the sample with a monoclonal antibody probe reagent may include the step of covering the sample with a Goat Anti-Mouse antibody probe reagent for approximately 20 minutes and covering the sample with a horseradish peroxidase may include the step of covering the sample with a horseradish peroxidase for approximately 20 minutes.

[0021] In another exemplary embodiment of the invention, the step f) of covering the sample with a primary SPARC antibody and primary HSP90 includes covering the sample with a polyclonal antibody and the step of covering the sample with a secondary detection reagent includes covering the sample with a secondary antibody reagent. Covering the sample with a secondary antibody reagent may include covering the sample with a goat anti-rat immunoglobulin G (IgG) reagent for approximately 15 minutes and covering the sample with a horseradish peroxidase conjugate may include the step of covering the sample with a streptavidin horseradish peroxidase reagent for approximately 15 minutes.

[0022] In another embodiment, the step of detecting an elevated level of SPARC includes examining the sample under a microscope and comparing the sample to at least one of a negative control for SPARC and a positive control for SPARC, and/or at least one of a negative control for HSP90 and a positive control for HSP90. This step may further include the step of assigning a value to the intensity of the staining for a sample

that has an elevated SPARC and/or HSP90 level. The invention may further include the step of assigning an overall score to a sample with an elevated SPARC and/or HSP90 level where the overall score is based on the intensity of staining and the percent of cells stained in the sample. Examples of overall scores may include a negative score, a moderately positive score, and a strongly positive score.

[0023] In still another exemplary embodiment of the invention, steps c) through p) above may be automated.

[0024] The present invention also provides a method for selecting a candidate treatment for a malignancy, comprising the steps of obtaining a sample of the malignancy, detecting whether the sample has an elevated level of SPARC and/or HSP90, and selecting a SPARC or HSP90 associated therapeutic, e.g., a SPARC or HSP90 inhibitor, as a candidate treatment for the malignancy if the sample has an elevated level of SPARC and/or HSP90, respectively. The levels of SPARC and/or HSP90 can be determined in accordance with steps a) through t) above. Other molecular profiling techniques can also be used to determine the levels of SPARC and/or HSP90, instead of or in addition to IHC. Such techniques are described herein, including without limitation FISH, microarray, PCR or sequencing methods and variations thereof. When multiple techniques are employed, a prioritized list of findings can be used, e.g., IHC results are used first, then FISH, then microarray, or any such prioritization as is appropriate. The candidate treatment can include without limitation a mitotic inhibitor, such as a taxane, vinca alkaloid, another cytotoxic agents or any combination thereof. In some embodiments, the mitotic inhibitor is paclitaxel, nab-paclitaxel or paclitaxel bound to albumin (e.g., ABRAXANE®). In some embodiments, the vinca alkaloid is vincristine, vinblastine, vindesine or vinorelbine. Because the molecular profiling methods of the invention identify SPARC and/or HSP90 as targets of one or more candidate therapeutic, any treatment associated with these targets can be selected as the the candidate therapeutic. Other SPARC and/or HSP90 associated therapeutics are described herein. The malignancy can be a malignancy of any tissue or any cancer described herein. In an embodiment, the malignancy is a sarcoma. The sarcoma can be an interdigitating dendritic cell sarcoma.

[0025] The method of the present invention for selecting a candidate treatment may also include the step of assigning an overall score to a sample having an elevated level of SPARC and/or HSP90 where the overall score is based on intensity of staining and the percent of cells stained in the sample, and the step of treating the malignancy with the candidate therapeutic may only be done when the tissue sample has an overall score that is strongly positive. For example, the staining intensity may be at least 2+ in more than 30% of the cells in the sample. Otherwise, the methods can include selecting other candidate therapeutics using the molecular profiling approaches presented herein.

[0026] The invention also provides methods for diagnosing or prognosing a malignancy. The method can be used to detect malignancy in any tissue, including without limitation in at least a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, an adipose tissue, and potentially any other mammalian tissue which includes the steps of obtaining a sample from at least one of a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, an adipose tissue, or any other mammalian tissue, detecting whether the sample has an elevated level of SPARC and/or HSP90, and determining that the sample is malignant if the sample has an elevated level of SPARC. In an embodiment, the malignancy is a sarcoma. The sarcoma can be an interdigitating dendritic cell sarcoma. The elevated levels can be determined using molecular profiling methods, such as in accordance with steps a) through t) above. The method may also include the step of assigning an overall score to a sample having

an elevated level of SPARC and/or HSP90 where the overall score is based on intensity of staining and the percent of cells stained in the sample, and the step of determining that the sample is malignant is only made if the sample has an overall score that is strongly positive.

[0027] The invention also provides a method of assessing SPARC and HSP90 levels in a biological tissue comprising: performing immunohistochemistry on a tissue sample from a subject; and, determining whether SPARC and HSP90 are expressed in at least 30% of cells in the tissue sample, wherein SPARC and HSP90 expression in at least 30% of cells is indicative of a disease such as a cancer. Methods of assessing SPARC and HSP90 levels in a biological tissue can also comprise performing immunohistochemistry with a monoclonal or a polyclonal SPARC antibody and a monoclonal or a polyclonal HSP90 antibody on a tissue sample from a subject; determining whether the monoclonal or polyclonal SPARC antibody associates with SPARC polypeptide or a fragment thereof in the sample, and determining whether the monoclonal or polyclonal HSP90 antibody associates with HSP90 polypeptide or a fragment thereof in the sample wherein an association of the monoclonal or polyclonal SPARC antibody with the SPARC polypeptide or fragment thereof and an association of the monoclonal or polyclonal HSP90 antibody with the HSP90 polypeptide or fragment thereof is indicative of a disease.

[0028] The invention also provides a method of diagnosing a sarcoma in a subject comprising: obtaining a biopsy; detecting SPARC; detecting HSP90; and comparing the level of the detected SPARC and HSP90 to an average level of SPARC and HSP in a control population, e.g., a population of individuals that not have a sarcoma. The method of diagnosing can be used for diagnosing interdigitating dendritic cell sarcoma. The detection can be performed using any appropriate molecular profiling methodology, including without limitation any variation of immunohistochemistry, microarrary, FISH, PCR and/or sequencing. For example, the comparison can be performed by comparing the intensity of immunohistochemical staining. A normal level of SPARC and HSP can be obtained from a population of peer group to the subject, including, but is not limited to, a population of the same ethnicity, same gender, and similar age group such as a population 10 years older or younger than the subject. The sarcoma sample can be obtained from any malignancy such as a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, and an adipose tissue. The sarcoma can be an interdigitating dendritic cell sarcoma.

[0029] The methods of the invention can be carried out in vitro. The invention further provides for the use of a reagent in carrying out any of the methods of the invention.

INCORPORATION BY REFERENCE

[0030] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0032] **FIG. 1** is a flowchart of an illustrative embodiment of a method for determining individualized medical intervention for a particular disease state that utilizes molecular profiling of a patient's biological specimen that is non disease specific;

[0033] **FIG. 2** is a flow chart depicting exemplary embodiments of the method of the present invention for detecting an elevated level of SPARC or HSP90 in a biological tissue;

[0034] **FIG. 3** is a series of photographs showing expression of SPARC in a tissue sample after carrying out the monoclonal and polyclonal methods of the present invention for detecting an elevated level of SPARC compared to a positive control for SPARC and a negative control for SPARC;

[0035] **FIG. 4** is a table showing results of the exemplary monoclonal and polyclonal embodiments of the present invention for detecting an elevated level of SPARC in a sample set of biological tissues;

[0036] **FIG. 5** illustrates an exemplary scoring chart used for assigning an overall score to a tissue sample after carrying out the methods of the present invention for detecting an elevated level of SPARC or HSP90;

[0037] **FIG. 6** is a flow chart depicting an exemplary embodiment of a method for treating a malignancy in accordance with the present invention;

[0038] **FIG. 7** is a flow chart showing an exemplary embodiment of a method for diagnosing a malignancy in accordance with the present invention;

[0039] **FIG. 8** illustrates progression free survival (PFS) using therapy selected by molecular profiling (period B) with PFS for the most recent therapy on which the patient has just progressed (period A). If PFS(B) / PFS(A) ratio $\geq$ 1.3, then molecular profiling selected therapy was defined as having benefit for patient;

[0040] **FIG. 9** is a schematic of methods for identifying treatments by molecular profiling if a target is identified;

[0041] **FIG. 10** illustrates the distribution of the patients in the study as performed in Example 4;

[0042] **FIG. 11** is graph depicting the results of the study with patients having PFS ratio $\geq$ 1.3 was 18/66 (27%);

[0043] **FIG. 12** is a waterfall plot of all the patients for maximum % change of summed diameters of target lesions with respect to baseline diameter;

[0044] **FIG. 13** illustrates the relationship between what clinician selected as what she/he would use to treat the patient before knowing what the molecular profiling results suggested. There were no matches for the 18 patients with PFS ratio $\geq$ 1.3;

[0045] **FIG. 14** is a schematic of the overall survival for the 18 patients with PFS ratio $\geq$ 1.3 versus all 66 patients;

[0046] **FIG. 15** illustrates a molecular profiling system that performs analysis of a cancer sample using a variety of components that measure expression levels, chromosomal aberrations and mutations. The molecular "blueprint" of the cancer is used to generate a prioritized ranking of druggable targets in tumor and their associated therapies; and

[0047] **FIGS. 16A-16N** show an illustrative patient report based on molecular profiling.

DETAILED DESCRIPTION OF THE INVENTION

[0048] The present invention provides methods and systems for identifying therapeutic agents for use in treatments on an individualized basis by using molecular profiling. The molecular profiling approach provides a

method for selecting a candidate treatment for an individual that could favorably change the clinical course for the individual with a condition or disease, such as cancer. The molecular profiling approach provides clinical benefit for individuals, such as identifying drug target(s) that provide a longer progression free survival (PFS), longer disease free survival (DFS), longer overall survival (OS) or extended lifespan. Methods and systems of the invention are directed to molecular profiling of cancer on an individual basis that can provide alternatives for treatment that may be convention or alternative to conventional treatment regimens. For example, alternative treatment regimes can be selected through molecular profiling methods of the invention where, a disease is refractory to current therapies, e.g., after a cancer has developed resistance to a standard-of-care treatment. Illustrative schemes for using molecular profiling to identify a treatment regime are shown in **FIGS. 1** and **15**, each of which is described in further detail herein.

[0049] Molecular profiling can be performed by any known means for detecting a molecule in a biological sample. Molecular profiling comprises methods that include but are not limited to, nucleic acid sequencing, such as a DNA sequencing or mRNA sequencing; immunohistochemistry (IHC); in situ hybridization (ISH); fluorescent in situ hybridization (FISH); various types of microarray (mRNA expression arrays, protein arrays, etc); various types of sequencing (Sanger, pyrosequencing, etc); comparative genomic hybridization (CGH); NextGen sequencing; Northern blot; Southern blot; immunoassay; and any other appropriate technique to assay the presence or quantity of a biological molecule of interest. In various embodiments of the invention, any one or more of these methods can be used concurrently or subsequent to each other for assessing target genes disclosed herein.

[0050] Molecular profiling of individual samples is used to select one or more candidate treatments for a disorder in a subject, e.g., by identifying targets for drugs that may be effective for a given cancer. For example, the candidate treatment can be a treatment known to have an effect on cells that differentially express genes as identified by molecular profiling techniques, an experimental drug, a government or regulatory approved drug or any combination of such drugs, which may have been studied and approved for a particular indication that is the same as or different from the indication of the subject from whom a biological sample is obtain and molecularly profiled.

[0051] When multiple biomarker targets are revealed by assessing target genes by molecular profiling, one or more decision rules can be put in place to prioritize the selection of certain therapeutic agent for treatment of an individual on a personalized basis. Rules of the invention aide prioritizing treatment, e.g., direct results of molecular profiling, anticipated efficacy of therapeutic agent, prior history with the same or other treatments, expected side effects, availability of therapeutic agent, cost of therapeutic agent, drug-drug interactions, and other factors considered by a treating physician. Based on the recommended and prioritized therapeutic agent targets, a physician can decide on the course of treatment for a particular individual. Accordingly, molecular profiling methods and systems of the invention can select candidate treatments based on individual characteristics of diseased cells, e.g., tumor cells, and other personalized factors in a subject in need of treatment, as opposed to relying on a traditional one-size fits all approach that is conventionally used to treat individuals suffering from a disease, especially cancer. In some cases, the recommended treatments are those not typically used to treat the disease or disorder inflicting the subject. In some cases, the recommended treatments are used after standard-of-care therapies are no longer providing adequate efficacy.

[0052] Biological Entities

[0053] Nucleic acids include deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, or complements thereof. Nucleic acids can contain known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs). Nucleic acid sequence can encompass conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); Rossolini et al., Mol. Cell Probes 8:91-98 (1994)). The term nucleic acid can be used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0054] A particular nucleic acid sequence may implicitly encompass the particular sequence and "splice variants" and nucleic acid sequences encoding truncated forms. Similarly, a particular protein encoded by a nucleic acid can encompass any protein encoded by a splice variant or truncated form of that nucleic acid. "Splice variants," as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition. Nucleic acids can be truncated at the 5' end or at the 3' end. Polypeptides can be truncated at the N-terminal end or the C-terminal end. Truncated versions of nucleic acid or polypeptide sequences can be naturally occurring or created using recombinant techniques.

[0055] The terms "genetic variant" and "nucleotide variant" are used herein interchangeably to refer to changes or alterations to the reference human gene or cDNA sequence at a particular locus, including, but not limited to, nucleotide base deletions, insertions, inversions, and substitutions in the coding and non-coding regions. Deletions may be of a single nucleotide base, a portion or a region of the nucleotide sequence of the gene, or of the entire gene sequence. Insertions may be of one or more nucleotide bases. The genetic variant or nucleotide variant may occur in transcriptional regulatory regions, untranslated regions of mRNA, exons, introns, exon/intron junctions, etc. The genetic variant or nucleotide variant can potentially result in stop codons, frame shifts, deletions of amino acids, altered gene transcript splice forms or altered amino acid sequence.

[0056] An allele or gene allele comprises generally a naturally occurring gene having a reference sequence or a gene containing a specific nucleotide variant.

[0057] A haplotype refers to a combination of genetic (nucleotide) variants in a region of an mRNA or a genomic DNA on a chromosome found in an individual. Thus, a haplotype includes a number of genetically linked polymorphic variants which are typically inherited together as a unit.

[0058] As used herein, the term "amino acid variant" is used to refer to an amino acid change to a reference human protein sequence resulting from genetic variants or nucleotide variants to the reference human gene encoding the reference protein. The term "amino acid variant" is intended to encompass not only single amino

acid substitutions, but also amino acid deletions, insertions, and other significant changes of amino acid sequence in the reference protein.

[0059] The term "genotype" as used herein means the nucleotide characters at a particular nucleotide variant marker (or locus) in either one allele or both alleles of a gene (or a particular chromosome region). With respect to a particular nucleotide position of a gene of interest, the nucleotide(s) at that locus or equivalent thereof in one or both alleles form the genotype of the gene at that locus. A genotype can be homozygous or heterozygous. Accordingly, "genotyping" means determining the genotype, that is, the nucleotide(s) at a particular gene locus. Genotyping can also be done by determining the amino acid variant at a particular position of a protein which can be used to deduce the corresponding nucleotide variant(s).

[0060] The term "locus" refers to a specific position or site in a gene sequence or protein. Thus, there may be one or more contiguous nucleotides in a particular gene locus, or one or more amino acids at a particular locus in a polypeptide. Moreover, a locus may refer to a particular position in a gene where one or more nucleotides have been deleted, inserted, or inverted.

[0061] As used herein, the terms "polypeptide," "protein," and "peptide" are used interchangeably to refer to an amino acid chain in which the amino acid residues are linked by covalent peptide bonds. The amino acid chain can be of any length of at least two amino acids, including full-length proteins. Unless otherwise specified, polypeptide, protein, and peptide also encompass various modified forms thereof, including but not limited to glycosylated forms, phosphorylated forms, etc. A polypeptide, protein or peptide can also be referred to as a gene product.

[0062] Lists of gene and gene products that can be assayed by molecular profiling techniques are presented herein. Lists of genes may be presented in the context of molecular profiling techniques that detect a gene product (e.g., an mRNA or protein). One of skill will understand that this implies detection of the gene product of the listed genes. Similarly, lists of gene products may be presented in the context of molecular profiling techniques that detect a gene sequence or copy number. One of skill will understand that this implies detection of the gene corresponding to the gene products, including as an example DNA encoding the gene products. As will be appreciated by those skilled in the art, a “biomarker” or “marker” comprises a gene and/or gene product depending on the context.

[0063] The terms "label" and "detectable label" can refer to any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, chemical or similar methods. Such labels include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., DYNABEADS™), fluorescent dyes (e.g., fluorescein, Texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and calorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc) beads. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and calorimetric labels are detected by simply visualizing the colored label. Labels can include, e.g., ligands that bind to labeled antibodies,

fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand. An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden Introduction to Immunocytochemistry, 2nd ed., Springer Verlag, NY (1997); and in Haugland Handbook of Fluorescent Probes and Research Chemicals, a combined handbook and catalogue Published by Molecular Probes, Inc. (1996).

[0064] Detectable labels include, but are not limited to, nucleotides (labeled or unlabelled), compomers, sugars, peptides, proteins, antibodies, chemical compounds, conducting polymers, binding moieties such as biotin, mass tags, calorimetric agents, light emitting agents, chemiluminescent agents, light scattering agents, fluorescent tags, radioactive tags, charge tags (electrical or magnetic charge), volatile tags and hydrophobic tags, biomolecules (e.g., members of a binding pair antibody/antigen, antibody/antibody, antibody/antibody fragment, antibody/antibody receptor, antibody/protein A or protein G, hapten/anti-hapten, biotin/avidin, biotin/streptavidin, folic acid/folate binding protein, vitamin B12/intrinsic factor, chemical reactive group/complementary chemical reactive group (e.g., sulfhydryl/maleimide, sulfhydryl/haloacetyl derivative, amine/isotriocyanate, amine/succinimidyl ester, and amine/sulfonyl halides) and the like.

[0065] The term "antibody" as used herein encompasses naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, (e.g., Fab', $F(ab')_2$, Fab, Fv and rIgG). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, Ill.). See also, e.g., Kuby, J., Immunology, 3.sup.rd Ed., W. H. Freeman & Co., New York (1998). Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art. See, e.g., Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Harlow and Lane, Antibodies, 511-52, Cold Spring Harbor Laboratory publications, New York, 1988; Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); Borrebaeck, Antibody Engineering, 2d ed. (Oxford University Press 1995); each of which is incorporated herein by reference.

[0066] Unless otherwise specified, antibodies can include both polyclonal and monoclonal antibodies. Antibodies also include genetically engineered forms such as chimeric antibodies (e.g., humanized murine antibodies) and heteroconjugate antibodies (e.g., bispecific antibodies). The term also refers to recombinant single chain Fv fragments (scFv). The term antibody also includes bivalent or bispecific molecules, diabodies, triabodies, and tetrabodies. Bivalent and bispecific molecules are described in, e.g., Kostelny et al. (1992) J Immunol 148:1547, Pack and Pluckthun (1992) Biochemistry 31:1579, Holliger et al. (1993) Proc Natl Acad Sci USA. 90:6444, Gruber et al. (1994) J Immunol:5368, Zhu et al. (1997) Protein Sci 6:781, Hu et al. (1997) Cancer Res. 56:3055, Adams et al. (1993) Cancer Res. 53:4026, and McCartney, et al. (1995) Protein Eng. 8:301.

[0067] Typically, an antibody has a heavy and light chain. Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). Light and heavy chain variable regions contain four framework regions interrupted by three hyper-variable regions, also called complementarity-

determining regions (CDRs). The extent of the framework regions and CDRs have been defined. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three dimensional spaces. The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found. References to V_H refer to the variable region of an immunoglobulin heavy chain of an antibody, including the heavy chain of an Fv, scFv, or Fab. References to V_L refer to the variable region of an immunoglobulin light chain, including the light chain of an Fv, scFv, dsFv or Fab.

[0068] The phrase "single chain Fv" or "scFv" refers to an antibody in which the variable domains of the heavy chain and of the light chain of a traditional two chain antibody have been joined to form one chain. Typically, a linker peptide is inserted between the two chains to allow for proper folding and creation of an active binding site. A "chimeric antibody" is an immunoglobulin molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[0069] A "humanized antibody" is an immunoglobulin molecule that contains minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework (FR) regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992)). Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species.

[0070] The terms "epitope" and "antigenic determinant" refer to a site on an antigen to which an antibody binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by

tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed (1996).

[0071] The terms "primer", "probe," and "oligonucleotide" are used herein interchangeably to refer to a relatively short nucleic acid fragment or sequence. They can comprise DNA, RNA, or a hybrid thereof, or chemically modified analog or derivatives thereof. Typically, they are single-stranded. However, they can also be double-stranded having two complementing strands which can be separated by denaturation. Normally, primers, probes and oligonucleotides have a length of from about 8 nucleotides to about 200 nucleotides, preferably from about 12 nucleotides to about 100 nucleotides, and more preferably about 18 to about 50 nucleotides. They can be labeled with detectable markers or modified using conventional manners for various molecular biological applications.

[0072] The term "isolated" when used in reference to nucleic acids (e.g., genomic DNAs, cDNAs, mRNAs, or fragments thereof) is intended to mean that a nucleic acid molecule is present in a form that is substantially separated from other naturally occurring nucleic acids that are normally associated with the molecule. Because a naturally existing chromosome (or a viral equivalent thereof) includes a long nucleic acid sequence, an isolated nucleic acid can be a nucleic acid molecule having only a portion of the nucleic acid sequence in the chromosome but not one or more other portions present on the same chromosome. More specifically, an isolated nucleic acid can include naturally occurring nucleic acid sequences that flank the nucleic acid in the naturally existing chromosome (or a viral equivalent thereof). An isolated nucleic acid can be substantially separated from other naturally occurring nucleic acids that are on a different chromosome of the same organism. An isolated nucleic acid can also be a composition in which the specified nucleic acid molecule is significantly enriched so as to constitute at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or at least 99% of the total nucleic acids in the composition.

[0073] An isolated nucleic acid can be a hybrid nucleic acid having the specified nucleic acid molecule covalently linked to one or more nucleic acid molecules that are not the nucleic acids naturally flanking the specified nucleic acid. For example, an isolated nucleic acid can be in a vector. In addition, the specified nucleic acid may have a nucleotide sequence that is identical to a naturally occurring nucleic acid or a modified form or mutein thereof having one or more mutations such as nucleotide substitution, deletion/insertion, inversion, and the like.

[0074] An isolated nucleic acid can be prepared from a recombinant host cell (in which the nucleic acids have been recombinantly amplified and/or expressed), or can be a chemically synthesized nucleic acid having a naturally occurring nucleotide sequence or an artificially modified form thereof.

[0075] The term "isolated polypeptide" as used herein is defined as a polypeptide molecule that is present in a form other than that found in nature. Thus, an isolated polypeptide can be a non-naturally occurring polypeptide. For example, an isolated polypeptide can be a "hybrid polypeptide." An isolated polypeptide can also be a polypeptide derived from a naturally occurring polypeptide by additions or deletions or substitutions of amino acids. An isolated polypeptide can also be a "purified polypeptide" which is used herein to mean a composition

or preparation in which the specified polypeptide molecule is significantly enriched so as to constitute at least 10% of the total protein content in the composition. A "purified polypeptide" can be obtained from natural or recombinant host cells by standard purification techniques, or by chemically synthesis, as will be apparent to skilled artisans.

[0076] The terms "hybrid protein," "hybrid polypeptide," "hybrid peptide," "fusion protein," "fusion polypeptide," and "fusion peptide" are used herein interchangeably to mean a non-naturally occurring polypeptide or isolated polypeptide having a specified polypeptide molecule covalently linked to one or more other polypeptide molecules that do not link to the specified polypeptide in nature. Thus, a "hybrid protein" may be two naturally occurring proteins or fragments thereof linked together by a covalent linkage. A "hybrid protein" may also be a protein formed by covalently linking two artificial polypeptides together. Typically but not necessarily, the two or more polypeptide molecules are linked or "fused" together by a peptide bond forming a single non-branched polypeptide chain.

[0077] The term "high stringency hybridization conditions," when used in connection with nucleic acid hybridization, includes hybridization conducted overnight at 42 °C in a solution containing 50% formamide, 5×SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate, pH 7.6, 5×Denhardt's solution, 10% dextran sulfate, and 20 microgram/ml denatured and sheared salmon sperm DNA, with hybridization filters washed in 0.1×SSC at about 65 °C. The term "moderate stringent hybridization conditions," when used in connection with nucleic acid hybridization, includes hybridization conducted overnight at 37 °C in a solution containing 50% formamide, 5×SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate, pH 7.6, 5×Denhardt's solution, 10% dextran sulfate, and 20 microgram/ml denatured and sheared salmon sperm DNA, with hybridization filters washed in 1×SSC at about 50 °C. It is noted that many other hybridization methods, solutions and temperatures can be used to achieve comparable stringent hybridization conditions as will be apparent to skilled artisans.

[0078] For the purpose of comparing two different nucleic acid or polypeptide sequences, one sequence (test sequence) may be described to be a specific percentage identical to another sequence (comparison sequence). The percentage identity can be determined by the algorithm of Karlin and Altschul, Proc. Natl. Acad. Sci. USA, 90:5873-5877 (1993), which is incorporated into various BLAST programs. The percentage identity can be determined by the "BLAST 2 Sequences" tool, which is available at the National Center for Biotechnology Information (NCBI) website. See Tatusova and Madden, FEMS Microbiol. Lett., 174(2):247-250 (1999). For pairwise DNA-DNA comparison, the BLASTN program is used with default parameters (e.g., Match: 1; Mismatch: -2; Open gap: 5 penalties; extension gap: 2 penalties; gap x_dropoff: 50; expect: 10; and word size: 11, with filter). For pairwise protein-protein sequence comparison, the BLASTP program can be employed using default parameters (e.g., Matrix: BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 15; expect: 10.0; and wordsize: 3, with filter). Percent identity of two sequences is calculated by aligning a test sequence with a comparison sequence using BLAST, determining the number of amino acids or nucleotides in the aligned test sequence that are identical to amino acids or nucleotides in the same position of the comparison sequence, and dividing the number of identical amino acids or nucleotides by the number of amino acids or nucleotides in the comparison sequence. When BLAST is used to compare two sequences, it aligns the sequences and yields the percent identity over defined, aligned regions. If the two sequences are aligned across their entire length, the percent identity yielded by the BLAST is the percent identity of the two sequences. If BLAST does not align the

two sequences over their entire length, then the number of identical amino acids or nucleotides in the unaligned regions of the test sequence and comparison sequence is considered to be zero and the percent identity is calculated by adding the number of identical amino acids or nucleotides in the aligned regions and dividing that number by the length of the comparison sequence. Various versions of the BLAST programs can be used to compare sequences, e.g., BLAST 2.1.2 or BLAST+ 2.2.22.

[0079] A subject or individual can be any animal which may benefit from the methods of the invention, including, e.g., humans and non-human mammals, such as primates, rodents, horses, dogs and cats. Subjects include without limitation a eukaryotic organisms, most preferably a mammal such as a primate, e.g., chimpanzee or human, cow; dog; cat; a rodent, e.g., guinea pig, rat, mouse; rabbit; or a bird; reptile; or fish. Subjects specifically intended for treatment using the methods described herein include humans. A subject may be referred to as an individual or a patient.

[0080] Treatment of a disease or individual according to the invention is an approach for obtaining beneficial or desired medical results, including clinical results, but not necessarily a cure. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. Treatment also includes prolonging survival as compared to expected survival if not receiving treatment or if receiving a different treatment. A treatment can include administration of a therapeutic agent, which can be an agent that exerts a cytotoxic, cytostatic, or immunomodulatory effect on diseased cells, e.g., cancer cells, or other cells that may promote a diseased state, e.g., activated immune cells. Therapeutic agents selected by the methods of the invention are not limited. Any therapeutic agent can be selected where a link can be made between molecular profiling and potential efficacy of the agent. Therapeutic agents include without limitation drugs, small molecules, protein therapies, antibody therapies, viral therapies, gene therapies, and the like. Cancer treatments or therapies include apoptosis-mediated and non-apoptosis mediated cancer therapies including, without limitation, chemotherapy, hormonal therapy, radiotherapy, immunotherapy, and combinations thereof. Chemotherapeutic agents comprise therapeutic agents and combinations of therapeutic agents that treat, cancer cells, e.g., by killing those cells. Examples of different types of chemotherapeutic drugs include without limitation alkylating agents (e.g., nitrogen mustard derivatives, ethylenimines, alkylsulfonates, hydrazines and triazines, nitrosureas, and metal salts), plant alkaloids (e.g., vinca alkaloids, taxanes, podophyllotoxins, and camptothecan analogs), antitumor antibiotics (e.g., anthracyclines, chromomycins, and the like), antimetabolites (e.g., folic acid antagonists, pyrimidine antagonists, purine antagonists, and adenosine deaminase inhibitors), topoisomerase I inhibitors, topoisomerase II inhibitors, and miscellaneous antineoplastics (e.g., ribonucleotide reductase inhibitors, adrenocortical steroid inhibitors, enzymes, antimicrotubule agents, and retinoids).

[0081] A biomarker refers generally to a molecule, including a gene or product thereof, nucleic acid, protein, carbohydrate structure, or glycolipid, characteristics of which can be detected in a tissue or cell to provide information that is predictive, diagnostic, prognostic and/or theranostic for sensitivity or resistance to candidate treatment.

[0082] Biological Samples

[0083] A sample as used herein includes any relevant biological sample that can be used for molecular profiling, e.g., sections of tissues such as biopsy or tissue removed during surgical or other procedures, bodily fluids, autopsy samples, and frozen sections taken for histological purposes. Such samples include blood and blood fractions or products (e.g., serum, buffy coat, plasma, platelets, red blood cells, and the like), sputum, cheek cells tissue, cultured cells (e.g., primary cultures, explants, and transformed cells), stool, urine, other biological or bodily fluids (e.g., prostatic fluid, gastric fluid, intestinal fluid, renal fluid, lung fluid, cerebrospinal fluid, and the like), etc. A sample may be processed according to techniques understood by those in the art. A sample can be without limitation fresh, frozen or fixed cells or tissue. In some embodiments, a sample comprises formalin-fixed paraffin-embedded (FFPE) tissue, fresh tissue or fresh frozen (FF) tissue. A sample can comprise cultured cells, including primary or immortalized cell lines derived from a subject sample. A sample can also refer to an extract from a sample from a subject. For example, a sample can comprise DNA, RNA or protein extracted from a tissue or a bodily fluid. Many techniques and commercial kits are available for such purposes. The fresh sample from the individual can be treated with an agent to preserve RNA prior to further processing, e.g., cell lysis and extraction. Samples can include frozen samples collected for other purposes. Samples can be associated with relevant information such as age, gender, and clinical symptoms present in the subject; source of the sample; and methods of collection and storage of the sample. A sample is typically obtained from a subject.

[0084] A biopsy comprises the process of removing a tissue sample for diagnostic or prognostic evaluation, and to the tissue specimen itself. Any biopsy technique known in the art can be applied to the molecular profiling methods of the present invention. The biopsy technique applied can depend on the tissue type to be evaluated (e.g., colon, prostate, kidney, bladder, lymph node, liver, bone marrow, blood cell, lung, breast, etc.), the size and type of the tumor (e.g., solid or suspended, blood or ascites), among other factors. Representative biopsy techniques include, but are not limited to, excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy, and bone marrow biopsy. An "excisional biopsy" refers to the removal of an entire tumor mass with a small margin of normal tissue surrounding it. An "incisional biopsy" refers to the removal of a wedge of tissue that includes a cross-sectional diameter of the tumor. Molecular profiling can use a "core-needle biopsy" of the tumor mass, or a "fine-needle aspiration biopsy" which generally obtains a suspension of cells from within the tumor mass. Biopsy techniques are discussed, for example, in Harrison's Principles of Internal Medicine, Kasper, et al., eds., 16th ed., 2005, Chapter 70, and throughout Part V.

[0085] Standard molecular biology techniques known in the art and not specifically described are generally followed as in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989), and as in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989) and as in Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988), and as in Watson et al., Recombinant DNA, Scientific American Books, New York and in Birren et al (eds) Genome Analysis: A Laboratory Manual Series, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) can be carried out generally as in PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, Calif. (1990).

[0086] Gene Expression Profiling

[0087] The methods and systems of the invention comprise expression profiling, which includes assessing differential expression of one or more target genes disclosed herein. Differential expression can include overexpression and/or underexpression of a biological product, e.g., a gene, mRNA or protein, compared to a control (or a reference). The control can include similar cells to the sample but without the disease (e.g., expression profiles obtained from samples from healthy individuals). A control can be a previously determined level that is indicative of a drug target efficacy associated with the particular disease and the particular drug target. The control can be derived from the same patient, e.g., a normal adjacent portion of the same organ as the diseased cells, the control can be derived from healthy tissues from other patients, or previously determined thresholds that are indicative of a disease responding or not-responding to a particular drug target. The control can also be a control found in the same sample, e.g. a housekeeping gene or a product thereof (e.g., mRNA or protein). For example, a control nucleic acid can be one which is known not to differ depending on the cancerous or non-cancerous state of the cell. The expression level of a control nucleic acid can be used to normalize signal levels in the test and reference populations. Illustrative control genes include, but are not limited to, e.g., β-actin, glyceraldehyde 3-phosphate dehydrogenase and ribosomal protein P1. Multiple controls or types of controls can be used. The source of differential expression can vary. For example, a gene copy number may be increased in a cell, thereby resulting in increased expression of the gene. Alternately, transcription of the gene may be modified, e.g., by chromatin remodeling, differential methylation, differential expression or activity of transcription factors, etc. Translation may also be modified, e.g., by differential expression of factors that degrade mRNA, translate mRNA, or silence translation, e.g., microRNAs or siRNAs. In some embodiments, differential expression comprises differential activity. For example, a protein may carry a mutation that increases the activity of the protein, such as constitutive activation, thereby contributing to a diseased state. Molecular profiling that reveals changes in activity can be used to guide treatment selection.

[0088] Methods of gene expression profiling include methods based on hybridization analysis of polynucleotides, and methods based on sequencing of polynucleotides. Commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting and in situ hybridization (Parker & Barnes (1999) Methods in Molecular Biology 106:247-283); RNAse protection assays (Hod (1992) Biotechniques 13:852-854); and reverse transcription polymerase chain reaction (RT-PCR) (Weis et al. (1992) Trends in Genetics 8:263-264). Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).

[0089] ***Reverse Transcriptase PCR (RT-PCR)***

[0090] RT-PCR can be used to determine RNA levels, e.g., mRNA or miRNA levels, of the biomarkers of the invention. RT-PCR can be used to compare such RNA levels of the biomarkers of the invention in different sample populations, in normal and tumor tissues, with or without drug treatment, to characterize patterns of gene expression, to discriminate between closely related RNAs, and to analyze RNA structure.

[0091] The first step is the isolation of RNA, e.g., mRNA, from a sample. The starting material can be total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines, respectively. Thus RNA can be isolated from a sample, e.g., tumor cells or tumor cell lines, and compared with

pooled DNA from healthy donors. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples.

[0092] General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al. (1997) Current Protocols of Molecular Biology, John Wiley and Sons. Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp & Locker (1987) Lab Invest. 56:A67, and De Andres et al., BioTechniques 18:42044 (1995). In particular, RNA isolation can be performed using purification kit, buffer set and protease from commercial manufacturers, such as Qiagen, according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Numerous RNA isolation kits are commercially available and can be used in the methods of the invention.

[0093] In the alternative, the first step is the isolation of miRNA from a target sample. The starting material is typically total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines, respectively. Thus RNA can be isolated from a variety of primary tumors or tumor cell lines, with pooled DNA from healthy donors. If the source of miRNA is a primary tumor, miRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples.

[0094] General methods for miRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al. (1997) Current Protocols of Molecular Biology, John Wiley and Sons. Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp & Locker (1987) Lab Invest. 56:A67, and De Andres et al., BioTechniques 18:42044 (1995). In particular, RNA isolation can be performed using purification kit, buffer set and protease from commercial manufacturers, such as Qiagen, according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Numerous RNA isolation kits are commercially available and can be used in the methods of the invention.

[0095] Whether the RNA comprises mRNA, miRNA or other types of RNA, gene expression profiling by RT-PCR can include reverse transcription of the RNA template into cDNA, followed by amplification in a PCR reaction. Commonly used reverse transcriptases include, but are not limited to, avilo myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

[0096] Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, it typically employs the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. TaqMan PCR typically utilizes the 5'-nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used. Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the

Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

[0097] TaqMan™ RT-PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700™ Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). In one specific embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700 Sequence Detection System. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optic cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

[0098] TaqMan data are initially expressed as Ct, or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (Ct).

[0099] To minimize errors and the effect of sample-to-sample variation, RT-PCR is usually performed using an internal standard. The ideal internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β-actin.

[00100] Real time quantitative PCR (also quantitative real time polymerase chain reaction, QRT-PCR or Q-PCR) is a more recent variation of the RT-PCR technique. Q-PCR can measure PCR product accumulation through a dual-labeled fluorigenic probe (i.e., TaqMan probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR. See, e.g. Held et al. (1996) Genome Research 6:986-994.

[00101] Protein-based detection techniques are also useful for molecular profiling, especially when the nucleotide variant causes amino acid substitutions or deletions or insertions or frame shift that affect the protein primary, secondary or tertiary structure. To detect the amino acid variations, protein sequencing techniques may be used. For example, a protein or fragment thereof corresponding to a gene can be synthesized by recombinant expression using a DNA fragment isolated from an individual to be tested. Preferably, a cDNA fragment of no more than 100 to 150 base pairs encompassing the polymorphic locus to be determined is used. The amino acid sequence of the peptide can then be determined by conventional protein sequencing methods. Alternatively, the HPLC-microscopy tandem mass spectrometry technique can be used for determining the amino acid sequence variations. In this technique, proteolytic digestion is performed on a protein, and the resulting peptide mixture is separated by reversed-phase chromatographic separation. Tandem mass spectrometry is then performed and the data collected is analyzed. See Gatlin et al., Anal. Chem., 72:757-763 (2000).

[00102] ***Microarray***

[00103] The biomarkers of the invention can also be identified, confirmed, and/or measured using the microarray technique. Thus, the expression profile biomarkers can be measured in cancer samples using

microarray technology. In this method, polynucleotide sequences of interest are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific DNA probes from cells or tissues of interest. The source of mRNA can be total RNA isolated from a sample, e.g., human tumors or tumor cell lines and corresponding normal tissues or cell lines. Thus RNA can be isolated from a variety of primary tumors or tumor cell lines. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples, which are routinely prepared and preserved in everyday clinical practice.

[00104] The expression profile of biomarkers can be measured in either fresh or paraffin-embedded tumor tissue, or body fluids using microarray technology. In this method, polynucleotide sequences of interest are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific DNA probes from cells or tissues of interest. As with the RT-PCR method, the source of miRNA typically is total RNA isolated from human tumors or tumor cell lines, including body fluids, such as serum, urine, tears, and exosomes and corresponding normal tissues or cell lines. Thus RNA can be isolated from a variety of sources. If the source of miRNA is a primary tumor, miRNA can be extracted, for example, from frozen tissue samples, which are routinely prepared and preserved in everyday clinical practice.

[00105] Also known as biochip, DNA chip, or gene array, cDNA microarray technology allows for identification of gene expression levels in a biologic sample. cDNAs or oligonucleotides, each representing a given gene, are immobilized on a substrate, e.g., a small chip, bead or nylon membrane, tagged, and serve as probes that will indicate whether they are expressed in biologic samples of interest. The simultaneous expression of thousands of genes can be monitored simultaneously.

[00106] In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate in a dense array. In one aspect, at least 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,500, 2,000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000 or at least 50,000 nucleotide sequences are applied to the substrate. Each sequence can correspond to a different gene, or multiple sequences can be arrayed per gene. The microarrayed genes, immobilized on the microchip, are suitable for hybridization under stringent conditions. Fluorescently labeled cDNA probes may be generated through incorporation of fluorescent nucleotides by reverse transcription of RNA extracted from tissues of interest. Labeled cDNA probes applied to the chip hybridize with specificity to each spot of DNA on the array. After stringent washing to remove non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. Quantitation of hybridization of each arrayed element allows for assessment of corresponding mRNA abundance. With dual color fluorescence, separately labeled cDNA probes generated from two sources of RNA are hybridized pairwise to the array. The relative abundance of the transcripts from the two sources corresponding to each specified gene is thus determined simultaneously. The miniaturized scale of the hybridization affords a convenient and rapid evaluation of the expression pattern for large numbers of genes. Such methods have been shown to have the sensitivity required to detect rare transcripts, which are expressed at a few copies per cell, and to reproducibly detect at least approximately two-fold differences in the expression levels (Schena et al. (1996) Proc. Natl. Acad. Sci. USA 93(2):106-149). Microarray analysis can be performed by commercially available equipment following manufacturer's protocols, including without limitation the Affymetrix GeneChip

technology (Affymetrix, Santa Clara, CA), Agilent (Agilent Technologies, Inc., Santa Clara, CA), or Illumina (Illumina, Inc., San Diego, CA) microarray technology.

[00107] The development of microarray methods for large-scale analysis of gene expression makes it possible to search systematically for molecular markers of cancer classification and outcome prediction in a variety of tumor types.

[00108] In some embodiments, the Agilent Whole Human Genome Microarray Kit (Agilent Technologies, Inc., Santa Clara, CA). The system can analyze more than 41,000 unique human genes and transcripts represented, all with public domain annotations. The system is used according to the manufacturer’s instructions.

[00109] In some embodiments, the Illumina Whole Genome DASL assay (Illumina Inc., San Diego, CA) is used. The system offers a method to simultaneously profile over 24,000 transcripts from minimal RNA input, from both fresh frozen (FF) and formalin-fixed paraffin embedded (FFPE) tissue sources, in a high throughput fashion.

[00110] Microarray expression analysis comprises identifying whether a gene or gene product is up-regulated or down-regulated relative to a reference. The identification can be performed using a statistical test to determine statistical significance of any differential expression observed. In some embodiments, statistical significance is determined using a parametric statistical test. The parametric statistical test can comprise, for example, a fractional factorial design, analysis of variance (ANOVA), a t-test, least squares, a Pearson correlation, simple linear regression, nonlinear regression, multiple linear regression, or multiple nonlinear regression. Alternatively, the parametric statistical test can comprise a one-way analysis of variance, two-way analysis of variance, or repeated measures analysis of variance. In other embodiments, statistical significance is determined using a nonparametric statistical test. Examples include, but are not limited to, a Wilcoxon signed-rank test, a Mann-Whitney test, a Kruskal-Wallis test, a Friedman test, a Spearman ranked order correlation coefficient, a Kendall Tau analysis, and a nonparametric regression test. In some embodiments, statistical significance is determined at a p-value of less than about 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001. Although the microarray systems used in the methods of the invention may assay thousands of transcripts, data analysis need only be performed on the transcripts of interest, thereby reducing the problem of multiple comparisons inherent in performing multiple statistical tests. The p-values can also be corrected for multiple comparisons, e.g., using a Bonferroni correction, a modification thereof, or other technique known to those in the art, e.g., the Hochberg correction, Holm-Bonferroni correction, Šidák correction, or Dunnett's correction. The degree of differential expression can also be taken into account. For example, a gene can be considered as differentially expressed when the fold-change in expression compared to control level is at least 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.5, 2.7, 3.0, 4, 5, 6, 7, 8, 9 or 10-fold different in the sample versus the control. The differential expression takes into account both overexpression and underexpression. A gene or gene product can be considered up or down-regulated if the differential expression meets a statistical threshold, a fold-change threshold, or both. For example, the criteria for identifying differential expression can comprise both a p-value of 0.001 and fold change of at least 1.5-fold (up or down). One of skill will understand that such statistical and threshold measures can be adapted to determine differential expression by any molecular profiling technique disclosed herein.

[00111] Various methods of the invention make use of many types of microarrays that detect the presence and potentially the amount of biological entities in a sample. Arrays typically contain addressable moieties that can

detect the presence of the entity in the sample, e.g., via a binding event. Microarrays include without limitation DNA microarrays, such as cDNA microarrays, oligonucleotide microarrays and SNP microarrays, microRNA arrays, protein microarrays, antibody microarrays, tissue microarrays, cellular microarrays (also called transfection microarrays), chemical compound microarrays, and carbohydrate arrays (glycoarrays). DNA arrays typically comprise addressable nucleotide sequences that can bind to sequences present in a sample. MicroRNA arrays, e.g., the MMChips array from the University of Louisville or commercial systems from Agilent, can be used to detect microRNAs. Protein microarrays can be used to identify protein–protein interactions, including without limitation identifying substrates of protein kinases, transcription factor protein-activation, or to identify the targets of biologically active small molecules. Protein arrays may comprise an array of different protein molecules, commonly antibodies, or nucleotide sequences that bind to proteins of interest. Antibody microarrays comprise antibodies spotted onto the protein chip that are used as capture molecules to detect proteins or other biological materials from a sample, e.g., from cell or tissue lysate solutions. For example, antibody arrays can be used to detect biomarkers from bodily fluids, e.g., serum or urine, for diagnostic applications. Tissue microarrays comprise separate tissue cores assembled in array fashion to allow multiplex histological analysis. Cellular microarrays, also called transfection microarrays, comprise various capture agents, such as antibodies, proteins, or lipids, which can interact with cells to facilitate their capture on addressable locations. Chemical compound microarrays comprise arrays of chemical compounds and can be used to detect protein or other biological materials that bind the compounds. Carbohydrate arrays (glycoarrays) comprise arrays of carbohydrates and can detect, e.g., protein that bind sugar moieties. One of skill will appreciate that similar technologies or improvements can be used according to the methods of the invention.

[00112] ***Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS)***

[00113] This method, described by Brenner et al. (2000) Nature Biotechnology 18:630-634, is a sequencing approach that combines non-gel-based signature sequencing with in vitro cloning of millions of templates on separate microbeads. First, a microbead library of DNA templates is constructed by in vitro cloning. This is followed by the assembly of a planar array of the template-containing microbeads in a flow cell at a high density. The free ends of the cloned templates on each microbead are analyzed simultaneously, using a fluorescence-based signature sequencing method that does not require DNA fragment separation. This method has been shown to simultaneously and accurately provide, in a single operation, hundreds of thousands of gene signature sequences from a cDNA library.

[00114] MPSS data has many uses. The expression levels of nearly all transcripts can be quantitatively determined; the abundance of signatures is representative of the expression level of the gene in the analyzed tissue. Quantitative methods for the analysis of tag frequencies and detection of differences among libraries have been published and incorporated into public databases for SAGE™ data and are applicable to MPSS data. The availability of complete genome sequences permits the direct comparison of signatures to genomic sequences and further extends the utility of MPSS data. Because the targets for MPSS analysis are not pre-selected (like on a microarray), MPSS data can characterize the full complexity of transcriptomes. This is analogous to sequencing millions of ESTs at once, and genomic sequence data can be used so that the source of the MPSS signature can be readily identified by computational means.

[00115] ***Serial Analysis of Gene Expression (SAGE)***

[00116] Serial analysis of gene expression (SAGE) is a method that allows the simultaneous and quantitative analysis of a large number of gene transcripts, without the need of providing an individual hybridization probe for each transcript. First, a short sequence tag (e.g., about 10-14 bp) is generated that contains sufficient information to uniquely identify a transcript, provided that the tag is obtained from a unique position within each transcript. Then, many transcripts are linked together to form long serial molecules, that can be sequenced, revealing the identity of the multiple tags simultaneously. The expression pattern of any population of transcripts can be quantitatively evaluated by determining the abundance of individual tags, and identifying the gene corresponding to each tag. See, e.g. Velculescu et al. (1995) Science 270:484-487; and Velculescu et al. (1997) Cell 88:243-51.

[00117] ***DNA Copy Number Profiling***

[00118] Any method capable of determining a DNA copy number profile of a particular sample can be used for molecular profiling according to the invention as long as the resolution is sufficient to identify the biomarkers of the invention. The skilled artisan is aware of and capable of using a number of different platforms for assessing whole genome copy number changes at a resolution sufficient to identify the copy number of the one or more biomarkers of the invention. Some of the platforms and techniques are described in the embodiments below.

[00119] In some embodiments, the copy number profile analysis involves amplification of whole genome DNA by a whole genome amplification method. The whole genome amplification method can use a strand displacing polymerase and random primers.

[00120] In some aspects of these embodiments, the copy number profile analysis involves hybridization of whole genome amplified DNA with a high density array. In a more specific aspect, the high density array has 5,000 or more different probes. In another specific aspect, the high density array has 5,000, 10,000, 20,000, 50,000, 100,000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, or 1,000,000 or more different probes. In another specific aspect, each of the different probes on the array is an oligonucleotide having from about 15 to 200 bases in length. In another specific aspect, each of the different probes on the array is an oligonucleotide having from about 15 to 200, 15 to 150, 15 to 100, 15 to 75, 15 to 60, or 20 to 55 bases in length.

[00121] In some embodiments, a microarray is employed to aid in determining the copy number profile for a sample, e.g., cells from a tumor. Microarrays typically comprise a plurality of oligomers (e.g., DNA or RNA polynucleotides or oligonucleotides, or other polymers), synthesized or deposited on a substrate (e.g., glass support) in an array pattern. The support-bound oligomers are "probes", which function to hybridize or bind with a sample material (e.g., nucleic acids prepared or obtained from the tumor samples), in hybridization experiments. The reverse situation can also be applied: the sample can be bound to the microarray substrate and the oligomer probes are in solution for the hybridization. In use, the array surface is contacted with one or more targets under conditions that promote specific, high-affinity binding of the target to one or more of the probes. In some configurations, the sample nucleic acid is labeled with a detectable label, such as a fluorescent tag, so that the hybridized sample and probes are detectable with scanning equipment. DNA array technology offers the potential of using a multitude (e.g., hundreds of thousands) of different oligonucleotides to analyze DNA copy number profiles. In some embodiments, the substrates used for arrays are surface-derivatized glass or silica, or polymer membrane surfaces (see e.g., in Z. Guo, et al., Nucleic Acids Res, 22, 5456-65 (1994); U. Maskos, E. M. Southern, Nucleic Acids Res, 20, 1679-84 (1992), and E. M. Southern, et al., Nucleic Acids Res, 22, 1368-

73 (1994), each incorporated by reference herein). Modification of surfaces of array substrates can be accomplished by many techniques. For example, siliceous or metal oxide surfaces can be derivatized with bifunctional silanes, i.e., silanes having a first functional group enabling covalent binding to the surface (e.g., Si-halogen or Si-alkoxy group, as in --$SiCl_3$ or --$Si(OCH_3)_3$, respectively) and a second functional group that can impart the desired chemical and/or physical modifications to the surface to covalently or non-covalently attach ligands and/or the polymers or monomers for the biological probe array. Silylated derivatizations and other surface derivatizations that are known in the art (see for example U.S. Pat. No. 5,624,711 to Sundberg, U.S. Pat. No. 5,266,222 to Willis, and U.S. Pat. No. 5,137,765 to Farnsworth, each incorporated by reference herein). Other processes for preparing arrays are described in U.S. Pat. No. 6,649,348, to Bass et. al., assigned to Agilent Corp., which disclose DNA arrays created by in situ synthesis methods.

[00122] Polymer array synthesis is also described extensively in the literature including in the following: WO 00/58516, U.S. Pat. Nos. 5,143,854, 5,242,974, 5,252,743, 5,324,633, 5,384,261, 5,405,783, 5,424,186, 5,451,683, 5,482,867, 5,491,074, 5,527,681, 5,550,215, 5,571,639, 5,578,832, 5,593,839, 5,599,695, 5,624,711, 5,631,734, 5,795,716, 5,831,070, 5,837,832, 5,856,101, 5,858,659, 5,936,324, 5,968,740, 5,974,164, 5,981,185, 5,981,956, 6,025,601, 6,033,860, 6,040,193, 6,090,555, 6,136,269, 6,269,846 and 6,428,752, 5,412,087, 6,147,205, 6,262,216, 6,310,189, 5,889,165, and 5,959,098 in PCT Applications Nos. PCT/US99/00730 (International Publication No. WO 99/36760) and PCT/US01/04285 (International Publication No. WO 01/58593), which are all incorporated herein by reference in their entirety for all purposes.

[00123] Nucleic acid arrays that are useful in the present invention include, but are not limited to, those that are commercially available from Affymetrix (Santa Clara, Calif.) under the brand name GeneChip™. Example arrays are shown on the website at affymetrix.com. Another microarray supplier is Illumina, Inc., of San Diego, Calif. with example arrays shown on their website at illumina.com.

[00124] In some embodiments, the inventive methods provide for sample preparation. Depending on the microarray and experiment to be performed, sample nucleic acid can be prepared in a number of ways by methods known to the skilled artisan. In some aspects of the invention, prior to or concurrent with genotyping (analysis of copy number profiles), the sample may be amplified any number of mechanisms. The most common amplification procedure used involves PCR. See, for example, PCR Technology: Principles and Applications for DNA Amplification (Ed. H. A. Erlich, Freeman Press, NY, N.Y., 1992); PCR Protocols: A Guide to Methods and Applications (Eds. Innis, et al., Academic Press, San Diego, Calif., 1990); Mattila et al., Nucleic Acids Res. 19, 4967 (1991); Eckert et al., PCR Methods and Applications 1, 17 (1991); PCR (Eds. McPherson et al., IRL Press, Oxford); and U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159 4,965,188, and 5,333,675, and each of which is incorporated herein by reference in their entireties for all purposes. In some embodiments, the sample may be amplified on the array (e.g., U.S. Pat. No. 6,300,070 which is incorporated herein by reference)

[00125] Other suitable amplification methods include the ligase chain reaction (LCR) (for example, Wu and Wallace, Genomics 4, 560 (1989), Landegren et al., Science 241, 1077 (1988) and Barringer et al. Gene 89:117 (1990)), transcription amplification (Kwoh et al., Proc. Natl. Acad. Sci. USA 86, 1173 (1989) and WO88/10315), self-sustained sequence replication (Guatelli et al., Proc. Nat. Acad. Sci. USA, 87, 1874 (1990) and WO90/06995), selective amplification of target polynucleotide sequences (U.S. Pat. No. 6,410,276), consensus sequence primed polymerase chain reaction (CP-PCR) (U.S. Pat. No. 4,437,975), arbitrarily primed polymerase chain reaction (AP-PCR) (U.S. Pat. Nos. 5,413,909, 5,861,245) and nucleic acid based sequence

amplification (NABSA). (See, U.S. Pat. Nos. 5,409,818, 5,554,517, and 6,063,603, each of which is incorporated herein by reference). Other amplification methods that may be used are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and in U.S. Ser. No. 09/854,317, each of which is incorporated herein by reference.

[00126] Additional methods of sample preparation and techniques for reducing the complexity of a nucleic sample are described in Dong et al., Genome Research 11, 1418 (2001), in U.S. Pat. Nos. 6,361,947, 6,391,592 and U.S. Ser. Nos. 09/916,135, 09/920,491 (U.S. Patent Application Publication 20030096235), 09/910,292 (U.S. Patent Application Publication 20030082543), and 10/013,598.

[00127] Methods for conducting polynucleotide hybridization assays are well developed in the art. Hybridization assay procedures and conditions used in the methods of the invention will vary depending on the application and are selected in accordance with the general binding methods known including those referred to in: Maniatis et al. Molecular Cloning: A Laboratory Manual (2.sup.nd Ed. Cold Spring Harbor, N.Y., 1989); Berger and Kimmel Methods in Enzymology, Vol. 152, Guide to Molecular Cloning Techniques (Academic Press, Inc., San Diego, Calif., 1987); Young and Davism, P.N.A.S, 80: 1194 (1983). Methods and apparatus for carrying out repeated and controlled hybridization reactions have been described in U.S. Pat. Nos. 5,871,928, 5,874,219, 6,045,996 and 6,386,749, 6,391,623 each of which are incorporated herein by reference.

[00128] The methods of the invention may also involve signal detection of hybridization between ligands in after (and/or during) hybridization. See U.S. Pat. Nos. 5,143,854, 5,578,832; 5,631,734; 5,834,758; 5,936,324; 5,981,956; 6,025,601; 6,141,096; 6,185,030; 6,201,639; 6,218,803; and 6,225,625, in U.S. Ser. No. 10/389,194 and in PCT Application PCT/US99/06097 (published as WO99/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

[00129] Methods and apparatus for signal detection and processing of intensity data are disclosed in, for example, U.S. Pat. Nos. 5,143,854, 5,547,839, 5,578,832, 5,631,734, 5,800,992, 5,834,758; 5,856,092, 5,902,723, 5,936,324, 5,981,956, 6,025,601, 6,090,555, 6,141,096, 6,185,030, 6,201,639; 6,218,803; and 6,225,625, in U.S. Ser. Nos. 10/389,194, 60/493,495 and in PCT Application PCT/US99/06097 (published as WO99/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

[00130] Immuno-based Assays

[00131] Protein-based detection molecular profiling techniques include immunoaffinity assays based on antibodies selectively immunoreactive with mutant gene encoded protein according to the present invention. These techniques include without limitation immunoprecipitation, Western blot analysis, molecular binding assays, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunofiltration assay (ELIFA), fluorescence activated cell sorting (FACS) and the like. For example, an optional method of detecting the expression of a biomarker in a sample comprises contacting the sample with an antibody against the biomarker, or an immunoreactive fragment of the antibody thereof, or a recombinant protein containing an antigen binding region of an antibody against the biomarker; and then detecting the binding of the biomarker in the sample. Methods for producing such antibodies are known in the art. Antibodies can be used to immunoprecipitate specific proteins from solution samples or to immunoblot proteins separated by, e.g., polyacrylamide gels. Immunocytochemical methods can also be used in detecting specific protein polymorphisms in tissues or cells. Other well-known antibody-based techniques can also be used including, e.g., ELISA, radioimmunoassay (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays

using monoclonal or polyclonal antibodies. See, e.g., U.S. Pat. Nos. 4,376,110 and 4,486,530, both of which are incorporated herein by reference.

[00132] In alternative methods, the sample may be contacted with an antibody specific for a biomarker under conditions sufficient for an antibody-biomarker complex to form, and then detecting the complex. The presence of the biomarker may be detected in a number of ways, such as by Western blotting and ELISA procedures for assaying a wide variety of tissues and samples, including plasma or serum. A wide range of immunoassay techniques using such an assay format are available, see, e.g., U.S. Pat. Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target biomarker.

[00133] A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate, and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of biomarker.

[00134] Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In a typical forward sandwich assay, a first antibody having specificity for the biomarker is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable conditions (e.g. from room temperature to 40°C such as between 25°C and 32°C inclusive) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the biomarker. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the molecular marker.

[00135] An alternative method involves immobilizing the target biomarkers in the sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary

complex. The complex is detected by the signal emitted by the reporter molecule. By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

[00136] In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, β-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-molecular marker complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of biomarker which was present in the sample. Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-molecular marker complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength, the fluorescence observed indicates the presence of the molecular marker of interest. Immunofluorescence and EIA techniques are both very well established in the art. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

[00137] ***Immunohistochemistry (IHC)***

[00138] IHC is a process of localizing antigens (e.g., proteins) in cells of a tissue binding antibodies specifically to antigens in the tissues. The antigen-binding antibody can be conjugated or fused to a tag that allows its detection, e.g., via visualization. In some embodiments, the tag is an enzyme that can catalyze a color-producing reaction, such as alkaline phosphatase or horseradish peroxidase. The enzyme can be fused to the antibody or non-covalently bound, e.g., using a biotin-avadin system. Alternatively, the antibody can be tagged with a fluorophore, such as fluorescein, rhodamine, DyLight Fluor or Alexa Fluor. The antigen-binding antibody can be directly tagged or it can itself be recognized by a detection antibody that carries the tag. Using IHC, one or more proteins may be detected. The expression of a gene product can be related to its staining intensity compared to control levels. In some embodiments, the gene product is considered differentially expressed if its staining varies at least 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.5, 2.7, 3.0, 4, 5, 6, 7, 8, 9 or 10-fold in the sample versus the control.

[00139] IHC comprises the application of antigen-antibody interactions to histochemical techniques. In an illustrative example, a tissue section is mounted on a slide and is incubated with antibodies (polyclonal or monoclonal) specific to the antigen (primary reaction). The antigen-antibody signal is then amplified using a second antibody conjugated to a complex of peroxidase antiperoxidase (PAP), avidin-biotin-peroxidase (ABC) or avidin-biotin alkaline phosphatase. In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding. Immunofluorescence is an alternate approach to visualize antigens. In this technique, the primary antigen-antibody signal is amplified using a second antibody conjugated to a fluorochrome. On UV light absorption, the fluorochrome emits its own light at a longer wavelength (fluorescence), thus allowing localization of antibody-antigen complexes.

[00140] Epigenetic Status

[00141] Molecular profiling methods according to the invention also comprise measuring epigenetic change, i.e., modification in a gene caused by an epigenetic mechanism, such as a change in methylation status or histone acetylation. Frequently, the epigenetic change will result in an alteration in the levels of expression of the gene which may be detected (at the RNA or protein level as appropriate) as an indication of the epigenetic change. Often the epigenetic change results in silencing or down regulation of the gene, referred to as "epigenetic silencing." The most frequently investigated epigenetic change in the methods of the invention involves determining the DNA methylation status of a gene, where an increased level of methylation is typically associated with the relevant cancer (since it may cause down regulation of gene expression). Aberrant methylation, which may be referred to as hypermethylation, of the gene or genes can be detected. Typically, the methylation status is determined in suitable CpG islands which are often found in the promoter region of the gene(s). The term "methylation," "methylation state" or "methylation status" may refers to the presence or absence of 5-methylcytosine at one or a plurality of CpG dinucleotides within a DNA sequence. CpG dinucleotides are typically concentrated in the promoter regions and exons of human genes.

[00142] Diminished gene expression can be assessed in terms of DNA methylation status or in terms of expression levels as determined by the methylation status of the gene. One method to detect epigenetic silencing is to determine that a gene which is expressed in normal cells is less expressed or not expressed in tumor cells. Accordingly, the invention provides for a method of molecular profiling comprising detecting epigenetic silencing.

[00143] Various assay procedures to directly detect methylation are known in the art, and can be used in conjunction with the present invention. These assays rely onto two distinct approaches: bisulphite conversion based approaches and non-bisulphite based approaches. Non-bisulphite based methods for analysis of DNA methylation rely on the inability of methylation-sensitive enzymes to cleave methylation cytosines in their restriction. The bisulphite conversion relies on treatment of DNA samples with sodium bisulphite which converts unmethylated cytosine to uracil, while methylated cytosines are maintained (Furuichi Y, Wataya Y, Hayatsu H, Ukita T. Biochem Biophys Res Commun. 1970 Dec 9;41(5):1185-91). This conversion results in a change in the sequence of the original DNA. Methods to detect such changes include MS AP-PCR (Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction), a technology that allows for a global scan of the genome using CG-rich primers to focus on the regions most likely to contain CpG dinucleotides, and described by Gonzalgo et al., Cancer Research 57:594-599, 1997; MethyLight™, which refers to the art-recognized fluorescence-based real-time PCR technique described by Eads et al., Cancer Res. 59:2302-2306,

1999; the HeavyMethyl™assay, in the embodiment thereof implemented herein, is an assay, wherein methylation specific blocking probes (also referred to herein as blockers) covering CpG positions between, or covered by the amplification primers enable methylation-specific selective amplification of a nucleic acid sample; HeavyMethyl™MethyLight™ is a variation of the MethyLight™ assay wherein the MethyLight™ assay is combined with methylation specific blocking probes covering CpG positions between the amplification primers; Ms-SNuPE (Methylation-sensitive Single Nucleotide Primer Extension) is an assay described by Gonzalgo & Jones, Nucleic Acids Res. 25:2529-2531, 1997; MSP (Methylation-specific PCR) is a methylation assay described by Herman et al. Proc. Natl. Acad. Sci. USA 93:9821-9826, 1996, and by U.S. Pat. No. 5,786,146; COBRA (Combined Bisulfite Restriction Analysis) is a methylation assay described by Xiong & Laird, Nucleic Acids Res. 25:2532-2534, 1997; MCA (Methylated CpG Island Amplification) is a methylation assay described by Toyota et al., Cancer Res. 59:2307-12, 1999, and in WO 00/26401A1.

[00144] Other techniques for DNA methylation analysis include sequencing, methylation-specific PCR (MS-PCR), melting curve methylation-specific PCR (McMS-PCR), MLPA with or without bisulfite treatment, QAMA, MSRE-PCR, MethyLight, ConLight-MSP, bisulfite conversion-specific methylation-specific PCR (BS-MSP), COBRA (which relies upon use of restriction enzymes to reveal methylation dependent sequence differences in PCR products of sodium bisulfite-treated DNA), methylation-sensitive single-nucleotide primer extension conformation (MS-SNuPE), methylation-sensitive single-strand conformation analysis (MS-SSCA), Melting curve combined bisulfite restriction analysis (McCOBRA), PyroMethA, HeavyMethyl, MALDI-TOF, MassARRAY, Quantitative analysis of methylated alleles (QAMA), enzymatic regional methylation assay (ERMA), QBSUPT, MethylQuant, Quantitative PCR sequencing and oligonucleotide-based microarray systems, Pyrosequencing, Meth-DOP-PCR. A review of some useful techniques is provided in Nucleic acids research, 1998, Vol. 26, No. 10, 2255-2264; Nature Reviews, 2003, Vol.3, 253-266; Oral Oncology, 2006, Vol. 42, 5-13, which references are incorporated herein in their entirety. Any of these techniques may be utilized in accordance with the present invention, as appropriate. Other techniques are described in U.S. Patent Publications 20100144836; and 20100184027, which applications are incorporated herein by reference in their entirety.

[00145] Through the activity of various acetylases and deacetylylases the DNA binding function of histone proteins is tightly regulated. Furthermore, histone acetylation and histone deactelyation have been linked with malignant progression. See Nature, 429: 457-63, 2004. Methods to analyze histone acetylation are described in U.S. Patent Publications 20100144543 and 20100151468, which applications are incorporated herein by reference in their entirety.

[00146] Sequence Analysis

[00147] Molecular profiling according to the present invention comprises methods for genotyping one or more biomarkers by determining whether an individual has one or more nucleotide variants (or amino acid variants) in one or more of the genes or gene products. Genotyping one or more genes according to the methods of the invention in some embodiments, can provide more evidence for selecting a treatment.

[00148] The biomarkers of the invention can be analyzed by any method useful for determining alterations in nucleic acids or the proteins they encode. According to one embodiment, the ordinary skilled artisan can analyze the one or more genes for mutations including deletion mutants, insertion mutants, frame shift mutants, nonsense mutants, missense mutant, and splice mutants.

[00149] Nucleic acid used for analysis of the one or more genes can be isolated from cells in the sample according to standard methodologies (Sambrook et al., 1989). The nucleic acid, for example, may be genomic DNA or fractionated or whole cell RNA, or miRNA acquired from exosomes or cell surfaces. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA; in another, it is exosomal RNA. Normally, the nucleic acid is amplified. Depending on the format of the assay for analyzing the one or more genes, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (e.g., ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

[00150] Various types of defects are known to occur in the biomarkers of the invention. Alterations include without limitation deletions, insertions, point mutations, and duplications. Point mutations can be silent or can result in stop codons, frame shift mutations or amino acid substitutions. Mutations in and outside the coding region of the one or more genes may occur and can be analyzed according to the methods of the invention. The target site of a nucleic acid of interest can include the region wherein the sequence varies. Examples include, but are not limited to, polymorphisms which exist in different forms such as single nucleotide variations, nucleotide repeats, multibase deletion (more than one nucleotide deleted from the consensus sequence), multibase insertion (more than one nucleotide inserted from the consensus sequence), microsatellite repeats (small numbers of nucleotide repeats with a typical 5-1000 repeat units), di-nucleotide repeats, tri-nucleotide repeats, sequence rearrangements (including translocation and duplication), chimeric sequence (two sequences from different gene origins are fused together), and the like. Among sequence polymorphisms, the most frequent polymorphisms in the human genome are single-base variations, also called single-nucleotide polymorphisms (SNPs). SNPs are abundant, stable and widely distributed across the genome.

[00151] Molecular profiling includes methods for haplotyping one or more genes. The haplotype is a set of genetic determinants located on a single chromosome and it typically contains a particular combination of alleles (all the alternative sequences of a gene) in a region of a chromosome. In other words, the haplotype is phased sequence information on individual chromosomes. Very often, phased SNPs on a chromosome define a haplotype. A combination of haplotypes on chromosomes can determine a genetic profile of a cell. It is the haplotype that determines a linkage between a specific genetic marker and a disease mutation. Haplotyping can be done by any methods known in the art. Common methods of scoring SNPs include hybridization microarray or direct gel sequencing, reviewed in Landgren et al., Genome Research, 8:769-776, 1998. For example, only one copy of one or more genes can be isolated from an individual and the nucleotide at each of the variant positions is determined. Alternatively, an allele specific PCR or a similar method can be used to amplify only one copy of the one or more genes in an individual, and the SNPs at the variant positions of the present invention are determined. The Clark method known in the art can also be employed for haplotyping. A high throughput molecular haplotyping method is also disclosed in Tost et al., Nucleic Acids Res., 30(19):e96 (2002), which is incorporated herein by reference.

[00152] Thus, additional variant(s) that are in linkage disequilibrium with the variants and/or haplotypes of the present invention can be identified by a haplotyping method known in the art, as will be apparent to a skilled

artisan in the field of genetics and haplotyping. The additional variants that are in linkage disequilibrium with a variant or haplotype of the present invention can also be useful in the various applications as described below.

[00153] For purposes of genotyping and haplotyping, both genomic DNA and mRNA/cDNA can be used, and both are herein referred to generically as "gene."

[00154] Numerous techniques for detecting nucleotide variants are known in the art and can all be used for the method of this invention. The techniques can be protein-based or nucleic acid-based. In either case, the techniques used must be sufficiently sensitive so as to accurately detect the small nucleotide or amino acid variations. Very often, a probe is utilized which is labeled with a detectable marker. Unless otherwise specified in a particular technique described below, any suitable marker known in the art can be used, including but not limited to, radioactive isotopes, fluorescent compounds, biotin which is detectable using streptavidin, enzymes (e.g., alkaline phosphatase), substrates of an enzyme, ligands and antibodies, etc. See Jablonski et al., Nucleic Acids Res., 14:6115-6128 (1986); Nguyen et al., Biotechniques, 13:116-123 (1992); Rigby et al., J. Mol. Biol., 113:237-251 (1977).

[00155] In a nucleic acid-based detection method, target DNA sample, i.e., a sample containing genomic DNA, cDNA, mRNA and/or miRNA, corresponding to the one or more genes must be obtained from the individual to be tested. Any tissue or cell sample containing the genomic DNA, miRNA, mRNA, and/or cDNA (or a portion thereof) corresponding to the one or more genes can be used. For this purpose, a tissue sample containing cell nucleus and thus genomic DNA can be obtained from the individual. Blood samples can also be useful except that only white blood cells and other lymphocytes have cell nucleus, while red blood cells are without a nucleus and contain only mRNA or miRNA. Nevertheless, miRNA and mRNA are also useful as either can be analyzed for the presence of nucleotide variants in its sequence or serve as template for cDNA synthesis. The tissue or cell samples can be analyzed directly without much processing. Alternatively, nucleic acids including the target sequence can be extracted, purified, and/or amplified before they are subject to the various detecting procedures discussed below. Other than tissue or cell samples, cDNAs or genomic DNAs from a cDNA or genomic DNA library constructed using a tissue or cell sample obtained from the individual to be tested are also useful.

[00156] To determine the presence or absence of a particular nucleotide variant, sequencing of the target genomic DNA or cDNA, particularly the region encompassing the nucleotide variant locus to be detected. Various sequencing techniques are generally known and widely used in the art including the Sanger method and Gilbert chemical method. The pyrosequencing method monitors DNA synthesis in real time using a luminometric detection system. Pyrosequencing has been shown to be effective in analyzing genetic polymorphisms such as single-nucleotide polymorphisms and can also be used in the present invention. See Nordstrom et al., Biotechnol. Appl. Biochem., 31(2):107-112 (2000); Ahmadian et al., Anal. Biochem., 280:103-110 (2000).

[00157] Nucleic acid variants can be detected by a suitable detection process. Non limiting examples of methods of detection, quantification, sequencing and the like are; mass detection of mass modified amplicons (e.g., matrix-assisted laser desorption ionization (MALDI) mass spectrometry and electrospray (ES) mass spectrometry), a primer extension method (e.g., iPLEX™; Sequenom, Inc.), microsequencing methods (e.g., a modification of primer extension methodology), ligase sequence determination methods (e.g., U.S. Pat. Nos. 5,679,524 and 5,952,174, and WO 01/27326), mismatch sequence determination methods (e.g., U.S. Pat. Nos. 5,851,770; 5,958,692; 6,110,684; and 6,183,958), direct DNA sequencing, restriction fragment length

polymorphism (RFLP analysis), allele specific oligonucleotide (ASO) analysis, methylation-specific PCR (MSPCR), pyrosequencing analysis, acycloprime analysis, Reverse dot blot, GeneChip microarrays, Dynamic allele-specific hybridization (DASH), Peptide nucleic acid (PNA) and locked nucleic acids (LNA) probes, TaqMan, Molecular Beacons, Intercalating dye, FRET primers, AlphaScreen, SNPstream, genetic bit analysis (GBA), Multiplex minisequencing, SNaPshot, GOOD assay, Microarray miniseq, arrayed primer extension (APEX), Microarray primer extension (e.g., microarray sequence determination methods), Tag arrays, Coded microspheres, Template-directed incorporation (TDI), fluorescence polarization, Colorimetric oligonucleotide ligation assay (OLA), Sequence-coded OLA, Microarray ligation, Ligase chain reaction, Padlock probes, Invader assay, hybridization methods (e.g., hybridization using at least one probe, hybridization using at least one fluorescently labeled probe, and the like), conventional dot blot analyses, single strand conformational polymorphism analysis (SSCP, e.g., U.S. Pat. Nos. 5,891,625 and 6,013,499; Orita et al., Proc. Natl. Acad. Sci. U.S.A. 86: 27776-2770 (1989)), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, and techniques described in Sheffield et al., Proc. Natl. Acad. Sci. USA 49: 699-706 (1991), White et al., Genomics 12: 301-306 (1992), Grompe et al., Proc. Natl. Acad. Sci. USA 86: 5855-5892 (1989), and Grompe, Nature Genetics 5: 111-117 (1993), cloning and sequencing, electrophoresis, the use of hybridization probes and quantitative real time polymerase chain reaction (QRT-PCR), digital PCR, nanopore sequencing, chips and combinations thereof. The detection and quantification of alleles or paralogs can be carried out using the "closed-tube" methods described in U.S. patent application Ser. No. 11/950,395, filed on Dec. 4, 2007. In some embodiments the amount of a nucleic acid species is determined by mass spectrometry, primer extension, sequencing (e.g., any suitable method, for example nanopore or pyrosequencing), Quantitative PCR (Q-PCR or QRT-PCR), digital PCR, combinations thereof, and the like.

[00158] The term "sequence analysis" as used herein refers to determining a nucleotide sequence, e.g., that of an amplification product. The entire sequence or a partial sequence of a polynucleotide, e.g., DNA or mRNA, can be determined, and the determined nucleotide sequence can be referred to as a “read” or “sequence read.” For example, linear amplification products may be analyzed directly without further amplification in some embodiments (e.g., by using single-molecule sequencing methodology). In certain embodiments, linear amplification products may be subject to further amplification and then analyzed (e.g., using sequencing by ligation or pyrosequencing methodology). Reads may be subject to different types of sequence analysis. Any suitable sequencing method can be utilized to detect, and determine the amount of, nucleotide sequence species, amplified nucleic acid species, or detectable products generated from the foregoing. Examples of certain sequencing methods are described hereafter.

[00159] A sequence analysis apparatus or sequence analysis component(s) includes an apparatus, and one or more components used in conjunction with such apparatus, that can be used by a person of ordinary skill to determine a nucleotide sequence resulting from processes described herein (e.g., linear and/or exponential amplification products). Examples of sequencing platforms include, without limitation, the 454 platform (Roche) (Margulies, M. et al. 2005 Nature 437, 376-380), Illumina Genomic Analyzer (or Solexa platform) or SOLID System (Applied Biosystems) or the Helicos True Single Molecule DNA sequencing technology (Harris TD et al. 2008 Science, 320, 106-109), the single molecule, real-time (SMRT™) technology of Pacific Biosciences, and nanopore sequencing (Soni G V and Meller A. 2007 Clin Chem 53: 1996-2001). Such platforms allow sequencing of many nucleic acid molecules isolated from a specimen at high orders of

multiplexing in a parallel manner (Dear Brief Funct Genomic Proteomic 2003; 1: 397-416). Each of these platforms allows sequencing of clonally expanded or non-amplified single molecules of nucleic acid fragments. Certain platforms involve, for example, sequencing by ligation of dye-modified probes (including cyclic ligation and cleavage), pyrosequencing, and single-molecule sequencing. Nucleotide sequence species, amplification nucleic acid species and detectable products generated there from can be analyzed by such sequence analysis platforms.

[00160] Sequencing by ligation is a nucleic acid sequencing method that relies on the sensitivity of DNA ligase to base-pairing mismatch. DNA ligase joins together ends of DNA that are correctly base paired. Combining the ability of DNA ligase to join together only correctly base paired DNA ends, with mixed pools of fluorescently labeled oligonucleotides or primers, enables sequence determination by fluorescence detection. Longer sequence reads may be obtained by including primers containing cleavable linkages that can be cleaved after label identification. Cleavage at the linker removes the label and regenerates the 5' phosphate on the end of the ligated primer, preparing the primer for another round of ligation. In some embodiments primers may be labeled with more than one fluorescent label, e.g., at least 1, 2, 3, 4, or 5 fluorescent labels.

[00161] Sequencing by ligation generally involves the following steps. Clonal bead populations can be prepared in emulsion microreactors containing target nucleic acid template sequences, amplification reaction components, beads and primers. After amplification, templates are denatured and bead enrichment is performed to separate beads with extended templates from undesired beads (e.g., beads with no extended templates). The template on the selected beads undergoes a 3' modification to allow covalent bonding to the slide, and modified beads can be deposited onto a glass slide. Deposition chambers offer the ability to segment a slide into one, four or eight chambers during the bead loading process. For sequence analysis, primers hybridize to the adapter sequence. A set of four color dye-labeled probes competes for ligation to the sequencing primer. Specificity of probe ligation is achieved by interrogating every 4th and 5th base during the ligation series. Five to seven rounds of ligation, detection and cleavage record the color at every 5th position with the number of rounds determined by the type of library used. Following each round of ligation, a new complimentary primer offset by one base in the 5' direction is laid down for another series of ligations. Primer reset and ligation rounds (5-7 ligation cycles per round) are repeated sequentially five times to generate 25-35 base pairs of sequence for a single tag. With mate-paired sequencing, this process is repeated for a second tag.

[00162] Pyrosequencing is a nucleic acid sequencing method based on sequencing by synthesis, which relies on detection of a pyrophosphate released on nucleotide incorporation. Generally, sequencing by synthesis involves synthesizing, one nucleotide at a time, a DNA strand complimentary to the strand whose sequence is being sought. Target nucleic acids may be immobilized to a solid support, hybridized with a sequencing primer, incubated with DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. Nucleotide solutions are sequentially added and removed. Correct incorporation of a nucleotide releases a pyrophosphate, which interacts with ATP sulfurylase and produces ATP in the presence of adenosine 5' phosphosulfate, fueling the luciferin reaction, which produces a chemiluminescent signal allowing sequence determination. The amount of light generated is proportional to the number of bases added. Accordingly, the sequence downstream of the sequencing primer can be determined. An illustrative system for pyrosequencing involves the following steps: ligating an adaptor nucleic acid to a nucleic acid under investigation and hybridizing the resulting nucleic acid to a bead; amplifying a nucleotide sequence in an emulsion; sorting beads

using a picoliter multiwell solid support; and sequencing amplified nucleotide sequences by pyrosequencing methodology (e.g., Nakano et al., "Single-molecule PCR using water-in-oil emulsion;" Journal of Biotechnology 102: 117-124 (2003)).

[00163] Certain single-molecule sequencing embodiments are based on the principal of sequencing by synthesis, and utilize single-pair Fluorescence Resonance Energy Transfer (single pair FRET) as a mechanism by which photons are emitted as a result of successful nucleotide incorporation. The emitted photons often are detected using intensified or high sensitivity cooled charge-couple-devices in conjunction with total internal reflection microscopy (TIRM). Photons are only emitted when the introduced reaction solution contains the correct nucleotide for incorporation into the growing nucleic acid chain that is synthesized as a result of the sequencing process. In FRET based single-molecule sequencing, energy is transferred between two fluorescent dyes, sometimes polymethine cyanine dyes Cy3 and Cy5, through long-range dipole interactions. The donor is excited at its specific excitation wavelength and the excited state energy is transferred, non-radiatively to the acceptor dye, which in turn becomes excited. The acceptor dye eventually returns to the ground state by radiative emission of a photon. The two dyes used in the energy transfer process represent the "single pair" in single pair FRET. Cy3 often is used as the donor fluorophore and often is incorporated as the first labeled nucleotide. Cy5 often is used as the acceptor fluorophore and is used as the nucleotide label for successive nucleotide additions after incorporation of a first Cy3 labeled nucleotide. The fluorophores generally are within 10 nanometers of each for energy transfer to occur successfully.

[00164] An example of a system that can be used based on single-molecule sequencing generally involves hybridizing a primer to a target nucleic acid sequence to generate a complex; associating the complex with a solid phase; iteratively extending the primer by a nucleotide tagged with a fluorescent molecule; and capturing an image of fluorescence resonance energy transfer signals after each iteration (e.g., U.S. Pat. No. 7,169,314; Braslavsky et al., PNAS 100(7): 3960-3964 (2003)). Such a system can be used to directly sequence amplification products (linearly or exponentially amplified products) generated by processes described herein. In some embodiments the amplification products can be hybridized to a primer that contains sequences complementary to immobilized capture sequences present on a solid support, a bead or glass slide for example. Hybridization of the primer-amplification product complexes with the immobilized capture sequences, immobilizes amplification products to solid supports for single pair FRET based sequencing by synthesis. The primer often is fluorescent, so that an initial reference image of the surface of the slide with immobilized nucleic acids can be generated. The initial reference image is useful for determining locations at which true nucleotide incorporation is occurring. Fluorescence signals detected in array locations not initially identified in the "primer only" reference image are discarded as non-specific fluorescence. Following immobilization of the primer-amplification product complexes, the bound nucleic acids often are sequenced in parallel by the iterative steps of, a) polymerase extension in the presence of one fluorescently labeled nucleotide, b) detection of fluorescence using appropriate microscopy, TIRM for example, c) removal of fluorescent nucleotide, and d) return to step a with a different fluorescently labeled nucleotide.

[00165] In some embodiments, nucleotide sequencing may be by solid phase single nucleotide sequencing methods and processes. Solid phase single nucleotide sequencing methods involve contacting target nucleic acid and solid support under conditions in which a single molecule of sample nucleic acid hybridizes to a single molecule of a solid support. Such conditions can include providing the solid support molecules and a single

molecule of target nucleic acid in a "microreactor." Such conditions also can include providing a mixture in which the target nucleic acid molecule can hybridize to solid phase nucleic acid on the solid support. Single nucleotide sequencing methods useful in the embodiments described herein are described in U.S. Provisional Patent Application Ser. No. 61/021,871 filed Jan. 17, 2008.

[00166] In certain embodiments, nanopore sequencing detection methods include (a) contacting a target nucleic acid for sequencing ("base nucleic acid," e.g., linked probe molecule) with sequence-specific detectors, under conditions in which the detectors specifically hybridize to substantially complementary subsequences of the base nucleic acid; (b) detecting signals from the detectors and (c) determining the sequence of the base nucleic acid according to the signals detected. In certain embodiments, the detectors hybridized to the base nucleic acid are disassociated from the base nucleic acid (e.g., sequentially dissociated) when the detectors interfere with a nanopore structure as the base nucleic acid passes through a pore, and the detectors disassociated from the base sequence are detected. In some embodiments, a detector disassociated from a base nucleic acid emits a detectable signal, and the detector hybridized to the base nucleic acid emits a different detectable signal or no detectable signal. In certain embodiments, nucleotides in a nucleic acid (e.g., linked probe molecule) are substituted with specific nucleotide sequences corresponding to specific nucleotides ("nucleotide representatives"), thereby giving rise to an expanded nucleic acid (e.g., U.S. Pat. No. 6,723,513), and the detectors hybridize to the nucleotide representatives in the expanded nucleic acid, which serves as a base nucleic acid. In such embodiments, nucleotide representatives may be arranged in a binary or higher order arrangement (e.g., Soni and Meller, Clinical Chemistry 53(11): 1996-2001 (2007)). In some embodiments, a nucleic acid is not expanded, does not give rise to an expanded nucleic acid, and directly serves a base nucleic acid (e.g., a linked probe molecule serves as a non-expanded base nucleic acid), and detectors are directly contacted with the base nucleic acid. For example, a first detector may hybridize to a first subsequence and a second detector may hybridize to a second subsequence, where the first detector and second detector each have detectable labels that can be distinguished from one another, and where the signals from the first detector and second detector can be distinguished from one another when the detectors are disassociated from the base nucleic acid. In certain embodiments, detectors include a region that hybridizes to the base nucleic acid (e.g., two regions), which can be about 3 to about 100 nucleotides in length (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 nucleotides in length). A detector also may include one or more regions of nucleotides that do not hybridize to the base nucleic acid. In some embodiments, a detector is a molecular beacon. A detector often comprises one or more detectable labels independently selected from those described herein. Each detectable label can be detected by any convenient detection process capable of detecting a signal generated by each label (e.g., magnetic, electric, chemical, optical and the like). For example, a CD camera can be used to detect signals from one or more distinguishable quantum dots linked to a detector.

[00167] In certain sequence analysis embodiments, reads may be used to construct a larger nucleotide sequence, which can be facilitated by identifying overlapping sequences in different reads and by using identification sequences in the reads. Such sequence analysis methods and software for constructing larger sequences from reads are known to the person of ordinary skill (e.g., Venter et al., Science 291: 1304-1351 (2001)). Specific reads, partial nucleotide sequence constructs, and full nucleotide sequence constructs may be compared between nucleotide sequences within a sample nucleic acid (i.e., internal comparison) or may be compared with a

reference sequence (i.e., reference comparison) in certain sequence analysis embodiments. Internal comparisons can be performed in situations where a sample nucleic acid is prepared from multiple samples or from a single sample source that contains sequence variations. Reference comparisons sometimes are performed when a reference nucleotide sequence is known and an objective is to determine whether a sample nucleic acid contains a nucleotide sequence that is substantially similar or the same, or different, than a reference nucleotide sequence. Sequence analysis can be facilitated by the use of sequence analysis apparatus and components described above.

[00168] Primer extension polymorphism detection methods, also referred to herein as "microsequencing" methods, typically are carried out by hybridizing a complementary oligonucleotide to a nucleic acid carrying the polymorphic site. In these methods, the oligonucleotide typically hybridizes adjacent to the polymorphic site. The term "adjacent" as used in reference to "microsequencing" methods, refers to the 3' end of the extension oligonucleotide being sometimes 1 nucleotide from the 5' end of the polymorphic site, often 2 or 3, and at times 4, 5, 6, 7, 8, 9, or 10 nucleotides from the 5' end of the polymorphic site, in the nucleic acid when the extension oligonucleotide is hybridized to the nucleic acid. The extension oligonucleotide then is extended by one or more nucleotides, often 1, 2, or 3 nucleotides, and the number and/or type of nucleotides that are added to the extension oligonucleotide determine which polymorphic variant or variants are present. Oligonucleotide extension methods are disclosed, for example, in U.S. Pat. Nos. 4,656,127; 4,851,331; 5,679,524; 5,834,189; 5,876,934; 5,908,755; 5,912,118; 5,976,802; 5,981,186; 6,004,744; 6,013,431; 6,017,702; 6,046,005; 6,087,095; 6,210,891; and WO 01/20039. The extension products can be detected in any manner, such as by fluorescence methods (see, e.g., Chen & Kwok, Nucleic Acids Research 25: 347-353 (1997) and Chen et al., Proc. Natl. Acad. Sci. USA 94/20: 10756-10761 (1997)) or by mass spectrometric methods (e.g., MALDI-TOF mass spectrometry) and other methods described herein. Oligonucleotide extension methods using mass spectrometry are described, for example, in U.S. Pat. Nos. 5,547,835; 5,605,798; 5,691,141; 5,849,542; 5,869,242; 5,928,906; 6,043,031; 6,194,144; and 6,258,538.

Microsequencing detection methods often incorporate an amplification process that proceeds the extension step. The amplification process typically amplifies a region from a nucleic acid sample that comprises the polymorphic site. Amplification can be carried out utilizing methods described above, or for example using a pair of oligonucleotide primers in a polymerase chain reaction (PCR), in which one oligonucleotide primer typically is complementary to a region 3' of the polymorphism and the other typically is complementary to a region 5' of the polymorphism. A PCR primer pair may be used in methods disclosed in U.S. Pat. Nos. 4,683,195; 4,683,202, 4,965,188; 5,656,493; 5,998,143; 6,140,054; WO 01/27327; and WO 01/27329 for example. PCR primer pairs may also be used in any commercially available machines that perform PCR, such as any of the GeneAmp™ Systems available from Applied Biosystems.

[00169] Other appropriate sequencing methods include multiplex polony sequencing (as described in Shendure et al., Accurate Multiplex Polony Sequencing of an Evolved Bacterial Genome, Sciencexpress, Aug. 4, 2005, pg 1 available at www.sciencexpress.org/4 Aug. 2005/Page1/10.1126/science.1117389, incorporated herein by reference), which employs immobilized microbeads, and sequencing in microfabricated picoliter reactors (as described in Margulies et al., Genome Sequencing in Microfabricated High-Density Picolitre Reactors, Nature, August 2005, available at www.nature.com/nature (published online 31 Jul. 2005, doi:10.1038/nature03959, incorporated herein by reference).

[00170] Whole genome sequencing may also be utilized for discriminating alleles of RNA transcripts, in some embodiments. Examples of whole genome sequencing methods include, but are not limited to, nanopore-based sequencing methods, sequencing by synthesis and sequencing by ligation, as described above.

[00171] Nucleic acid variants can also be detected using standard electrophoretic techniques. Although the detection step can sometimes be preceded by an amplification step, amplification is not required in the embodiments described herein. Examples of methods for detection and quantification of a nucleic acid using electrophoretic techniques can be found in the art. A non-limiting example comprises running a sample (e.g., mixed nucleic acid sample isolated from maternal serum, or amplification nucleic acid species, for example) in an agarose or polyacrylamide gel. The gel may be labeled (e.g., stained) with ethidium bromide (see, Sambrook and Russell, Molecular Cloning: A Laboratory Manual 3d ed., 2001). The presence of a band of the same size as the standard control is an indication of the presence of a target nucleic acid sequence, the amount of which may then be compared to the control based on the intensity of the band, thus detecting and quantifying the target sequence of interest. In some embodiments, restriction enzymes capable of distinguishing between maternal and paternal alleles may be used to detect and quantify target nucleic acid species. In certain embodiments, oligonucleotide probes specific to a sequence of interest are used to detect the presence of the target sequence of interest. The oligonucleotides can also be used to indicate the amount of the target nucleic acid molecules in comparison to the standard control, based on the intensity of signal imparted by the probe.

[00172] Sequence-specific probe hybridization can be used to detect a particular nucleic acid in a mixture or mixed population comprising other species of nucleic acids. Under sufficiently stringent hybridization conditions, the probes hybridize specifically only to substantially complementary sequences. The stringency of the hybridization conditions can be relaxed to tolerate varying amounts of sequence mismatch. A number of hybridization formats are known in the art, which include but are not limited to, solution phase, solid phase, or mixed phase hybridization assays. The following articles provide an overview of the various hybridization assay formats: Singer et al., Biotechniques 4:230, 1986; Haase et al., Methods in Virology, pp. 189-226, 1984; Wilkinson, In situ Hybridization, Wilkinson ed., IRL Press, Oxford University Press, Oxford; and Hames and Higgins eds., Nucleic Acid Hybridization: A Practical Approach, IRL Press, 1987.

[00173] Hybridization complexes can be detected by techniques known in the art. Nucleic acid probes capable of specifically hybridizing to a target nucleic acid (e.g., mRNA or DNA) can be labeled by any suitable method, and the labeled probe used to detect the presence of hybridized nucleic acids. One commonly used method of detection is autoradiography, using probes labeled with ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability, and half-lives of the selected isotopes. Other labels include compounds (e.g., biotin and digoxigenin), which bind to antiligands or antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. In some embodiments, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

[00174] Alternatively, the restriction fragment length polymorphism (RFLP) and AFLP method may be used for molecular profiling. If a nucleotide variant in the target DNA corresponding to the one or more genes results in the elimination or creation of a restriction enzyme recognition site, then digestion of the target DNA with that

particular restriction enzyme will generate an altered restriction fragment length pattern. Thus, a detected RFLP or AFLP will indicate the presence of a particular nucleotide variant.

[00175] Another useful approach is the single-stranded conformation polymorphism assay (SSCA), which is based on the altered mobility of a single-stranded target DNA spanning the nucleotide variant of interest. A single nucleotide change in the target sequence can result in different intramolecular base pairing pattern, and thus different secondary structure of the single-stranded DNA, which can be detected in a non-denaturing gel. See Orita et al., Proc. Natl. Acad. Sci. USA, 86:2776-2770 (1989). Denaturing gel-based techniques such as clamped denaturing gel electrophoresis (CDGE) and denaturing gradient gel electrophoresis (DGGE) detect differences in migration rates of mutant sequences as compared to wild-type sequences in denaturing gel. See Miller et al., Biotechniques, 5:1016-24 (1999); Sheffield et al., Am. J. Hum, Genet., 49:699-706 (1991); Wartell et al., Nucleic Acids Res., 18:2699-2705 (1990); and Sheffield et al., Proc. Natl. Acad. Sci. USA, 86:232-236 (1989). In addition, the double-strand conformation analysis (DSCA) can also be useful in the present invention. See Arguello et al., Nat. Genet., 18:192-194 (1998).

[00176] The presence or absence of a nucleotide variant at a particular locus in the one or more genes of an individual can also be detected using the amplification refractory mutation system (ARMS) technique. See e.g., European Patent No. 0,332,435; Newton et al., Nucleic Acids Res., 17:2503-2515 (1989); Fox et al., Br. J. Cancer, 77:1267-1274 (1998); Robertson et al., Eur. Respir. J., 12:477-482 (1998). In the ARMS method, a primer is synthesized matching the nucleotide sequence immediately 5' upstream from the locus being tested except that the 3'-end nucleotide which corresponds to the nucleotide at the locus is a predetermined nucleotide. For example, the 3'-end nucleotide can be the same as that in the mutated locus. The primer can be of any suitable length so long as it hybridizes to the target DNA under stringent conditions only when its 3'-end nucleotide matches the nucleotide at the locus being tested. Preferably the primer has at least 12 nucleotides, more preferably from about 18 to 50 nucleotides. If the individual tested has a mutation at the locus and the nucleotide therein matches the 3'-end nucleotide of the primer, then the primer can be further extended upon hybridizing to the target DNA template, and the primer can initiate a PCR amplification reaction in conjunction with another suitable PCR primer. In contrast, if the nucleotide at the locus is of wild type, then primer extension cannot be achieved. Various forms of ARMS techniques developed in the past few years can be used. See e.g., Gibson et al., Clin. Chem. 43:1336-1341 (1997).

[00177] Similar to the ARMS technique is the mini sequencing or single nucleotide primer extension method, which is based on the incorporation of a single nucleotide. An oligonucleotide primer matching the nucleotide sequence immediately 5' to the locus being tested is hybridized to the target DNA, mRNA or miRNA in the presence of labeled dideoxyribonucleotides. A labeled nucleotide is incorporated or linked to the primer only when the dideoxyribonucleotides matches the nucleotide at the variant locus being detected. Thus, the identity of the nucleotide at the variant locus can be revealed based on the detection label attached to the incorporated dideoxyribonucleotides. See Syvanen et al., Genomics, 8:684-692 (1990); Shumaker et al., Hum. Mutat., 7:346-354 (1996); Chen et al., Genome Res., 10:549-547 (2000).

[00178] Another set of techniques useful in the present invention is the so-called "oligonucleotide ligation assay" (OLA) in which differentiation between a wild-type locus and a mutation is based on the ability of two oligonucleotides to anneal adjacent to each other on the target DNA molecule allowing the two oligonucleotides joined together by a DNA ligase. See Landergren et al., Science, 241:1077-1080 (1988); Chen et al, Genome

Res., 8:549-556 (1998); Iannone et al., Cytometry, 39:131-140 (2000). Thus, for example, to detect a single-nucleotide mutation at a particular locus in the one or more genes, two oligonucleotides can be synthesized, one having the sequence just 5' upstream from the locus with its 3' end nucleotide being identical to the nucleotide in the variant locus of the particular gene, the other having a nucleotide sequence matching the sequence immediately 3' downstream from the locus in the gene. The oligonucleotides can be labeled for the purpose of detection. Upon hybridizing to the target gene under a stringent condition, the two oligonucleotides are subject to ligation in the presence of a suitable ligase. The ligation of the two oligonucleotides would indicate that the target DNA has a nucleotide variant at the locus being detected.

[00179] Detection of small genetic variations can also be accomplished by a variety of hybridization-based approaches. Allele-specific oligonucleotides are most useful. See Conner et al., Proc. Natl. Acad. Sci. USA, 80:278-282 (1983); Saiki et al, Proc. Natl. Acad. Sci. USA, 86:6230-6234 (1989). Oligonucleotide probes (allele-specific) hybridizing specifically to a gene allele having a particular gene variant at a particular locus but not to other alleles can be designed by methods known in the art. The probes can have a length of, e.g., from 10 to about 50 nucleotide bases. The target DNA and the oligonucleotide probe can be contacted with each other under conditions sufficiently stringent such that the nucleotide variant can be distinguished from the wild-type gene based on the presence or absence of hybridization. The probe can be labeled to provide detection signals. Alternatively, the allele-specific oligonucleotide probe can be used as a PCR amplification primer in an "allele-specific PCR" and the presence or absence of a PCR product of the expected length would indicate the presence or absence of a particular nucleotide variant.

[00180] Other useful hybridization-based techniques allow two single-stranded nucleic acids annealed together even in the presence of mismatch due to nucleotide substitution, insertion or deletion. The mismatch can then be detected using various techniques. For example, the annealed duplexes can be subject to electrophoresis. The mismatched duplexes can be detected based on their electrophoretic mobility that is different from the perfectly matched duplexes. See Cariello, Human Genetics, 42:726 (1988). Alternatively, in an RNase protection assay, a RNA probe can be prepared spanning the nucleotide variant site to be detected and having a detection marker. See Giunta et al., Diagn. Mol. Path., 5:265-270 (1996); Finkelstein et al., Genomics, 7:167-172 (1990); Kinszler et al., Science 251:1366-1370 (1991). The RNA probe can be hybridized to the target DNA or mRNA forming a heteroduplex that is then subject to the ribonuclease RNase A digestion. RNase A digests the RNA probe in the heteroduplex only at the site of mismatch. The digestion can be determined on a denaturing electrophoresis gel based on size variations. In addition, mismatches can also be detected by chemical cleavage methods known in the art. See e.g., Roberts et al., Nucleic Acids Res., 25:3377-3378 (1997).

[00181] In the mutS assay, a probe can be prepared matching the gene sequence surrounding the locus at which the presence or absence of a mutation is to be detected, except that a predetermined nucleotide is used at the variant locus. Upon annealing the probe to the target DNA to form a duplex, the E. coli mutS protein is contacted with the duplex. Since the mutS protein binds only to heteroduplex sequences containing a nucleotide mismatch, the binding of the mutS protein will be indicative of the presence of a mutation. See Modrich et al., Ann. Rev. Genet., 25:229-253 (1991).

[00182] A great variety of improvements and variations have been developed in the art on the basis of the above-described basic techniques which can be useful in detecting mutations or nucleotide variants in the present invention. For example, the "sunrise probes" or "molecular beacons" use the fluorescence resonance

energy transfer (FRET) property and give rise to high sensitivity. See Wolf et al., Proc. Nat. Acad. Sci. USA, 85:8790-8794 (1988). Typically, a probe spanning the nucleotide locus to be detected are designed into a hairpin-shaped structure and labeled with a quenching fluorophore at one end and a reporter fluorophore at the other end. In its natural state, the fluorescence from the reporter fluorophore is quenched by the quenching fluorophore due to the proximity of one fluorophore to the other. Upon hybridization of the probe to the target DNA, the 5' end is separated apart from the 3'-end and thus fluorescence signal is regenerated. See Nazarenko et al., Nucleic Acids Res., 25:2516-2521 (1997); Rychlik et al., Nucleic Acids Res., 17:8543-8551 (1989); Sharkey et al., Bio/Technology 12:506-509 (1994); Tyagi et al., Nat. Biotechnol., 14:303-308 (1996); Tyagi et al., Nat. Biotechnol., 16:49-53 (1998). The homo-tag assisted non-dimer system (HANDS) can be used in combination with the molecular beacon methods to suppress primer-dimer accumulation. See Brownie et al., Nucleic Acids Res., 25:3235-3241 (1997).

[00183] Dye-labeled oligonucleotide ligation assay is a FRET-based method, which combines the OLA assay and PCR. See Chen et al., Genome Res. 8:549-556 (1998). TaqMan is another FRET-based method for detecting nucleotide variants. A TaqMan probe can be oligonucleotides designed to have the nucleotide sequence of the gene spanning the variant locus of interest and to differentially hybridize with different alleles. The two ends of the probe are labeled with a quenching fluorophore and a reporter fluorophore, respectively. The TaqMan probe is incorporated into a PCR reaction for the amplification of a target gene region containing the locus of interest using Taq polymerase. As Taq polymerase exhibits 5'-3' exonuclease activity but has no 3'-5' exonuclease activity, if the TaqMan probe is annealed to the target DNA template, the 5'-end of the TaqMan probe will be degraded by Taq polymerase during the PCR reaction thus separating the reporting fluorophore from the quenching fluorophore and releasing fluorescence signals. See Holland et al., Proc. Natl. Acad. Sci. USA, 88:7276-7280 (1991); Kalinina et al., Nucleic Acids Res., 25:1999-2004 (1997); Whitcombe et al., Clin. Chem., 44:918-923 (1998).

[00184] In addition, the detection in the present invention can also employ a chemiluminescence-based technique. For example, an oligonucleotide probe can be designed to hybridize to either the wild-type or a variant gene locus but not both. The probe is labeled with a highly chemiluminescent acridinium ester. Hydrolysis of the acridinium ester destroys chemiluminescence. The hybridization of the probe to the target DNA prevents the hydrolysis of the acridinium ester. Therefore, the presence or absence of a particular mutation in the target DNA is determined by measuring chemiluminescence changes. See Nelson et al., Nucleic Acids Res., 24:4998-5003 (1996).

[00185] The detection of genetic variation in the gene in accordance with the present invention can also be based on the "base excision sequence scanning" (BESS) technique. The BESS method is a PCR-based mutation scanning method. BESS T-Scan and BESS G-Tracker are generated which are analogous to T and G ladders of dideoxy sequencing. Mutations are detected by comparing the sequence of normal and mutant DNA. See, e.g., Hawkins et al., Electrophoresis, 20:1171-1176 (1999).

[00186] Mass spectrometry can be used for molecular profiling according to the invention. See Graber et al., Curr. Opin. Biotechnol., 9:14-18 (1998). For example, in the primer oligo base extension (PROBE™) method, a target nucleic acid is immobilized to a solid-phase support. A primer is annealed to the target immediately 5' upstream from the locus to be analyzed. Primer extension is carried out in the presence of a selected mixture of

deoxyribonucleotides and dideoxyribonucleotides. The resulting mixture of newly extended primers is then analyzed by MALDI-TOF. See e.g., Monforte et al., Nat. Med., 3:360-362 (1997).

[00187] In addition, the microchip or microarray technologies are also applicable to the detection method of the present invention. Essentially, in microchips, a large number of different oligonucleotide probes are immobilized in an array on a substrate or carrier, e.g., a silicon chip or glass slide. Target nucleic acid sequences to be analyzed can be contacted with the immobilized oligonucleotide probes on the microchip. See Lipshutz et al., Biotechniques, 19:442-447 (1995); Chee et al., Science, 274:610-614 (1996); Kozal et al., Nat. Med. 2:753-759 (1996); Hacia et al., Nat. Genet., 14:441-447 (1996); Saiki et al., Proc. Natl. Acad. Sci. USA, 86:6230-6234 (1989); Gingeras et al., Genome Res., 8:435-448 (1998). Alternatively, the multiple target nucleic acid sequences to be studied are fixed onto a substrate and an array of probes is contacted with the immobilized target sequences. See Drmanac et al., Nat. Biotechnol., 16:54-58 (1998). Numerous microchip technologies have been developed incorporating one or more of the above described techniques for detecting mutations. The microchip technologies combined with computerized analysis tools allow fast screening in a large scale. The adaptation of the microchip technologies to the present invention will be apparent to a person of skill in the art apprised of the present disclosure. See, e.g., U.S. Pat. No. 5,925,525 to Fodor et al; Wilgenbus et al., J. Mol. Med., 77:761-786 (1999); Graber et al., Curr. Opin. Biotechnol., 9:14-18 (1998); Hacia et al., Nat. Genet., 14:441-447 (1996); Shoemaker et al., Nat. Genet., 14:450-456 (1996); DeRisi et al., Nat. Genet., 14:457-460 (1996); Chee et al., Nat. Genet., 14:610-614 (1996); Lockhart et al., Nat. Genet., 14:675-680 (1996); Drobyshev et al., Gene, 188:45-52 (1997).

[00188] As is apparent from the above survey of the suitable detection techniques, it may or may not be necessary to amplify the target DNA, i.e., the gene, cDNA, mRNA, miRNA, or a portion thereof to increase the number of target DNA molecule, depending on the detection techniques used. For example, most PCR-based techniques combine the amplification of a portion of the target and the detection of the mutations. PCR amplification is well known in the art and is disclosed in U.S. Pat. Nos. 4,683,195 and 4,800,159, both which are incorporated herein by reference. For non-PCR-based detection techniques, if necessary, the amplification can be achieved by, e.g., in vivo plasmid multiplication, or by purifying the target DNA from a large amount of tissue or cell samples. See generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989. However, even with scarce samples, many sensitive techniques have been developed in which small genetic variations such as single-nucleotide substitutions can be detected without having to amplify the target DNA in the sample. For example, techniques have been developed that amplify the signal as opposed to the target DNA by, e.g., employing branched DNA or dendrimers that can hybridize to the target DNA. The branched or dendrimer DNAs provide multiple hybridization sites for hybridization probes to attach thereto thus amplifying the detection signals. See Detmer et al., J. Clin. Microbiol., 34:901-907 (1996); Collins et al., Nucleic Acids Res., 25:2979-2984 (1997); Horn et al., Nucleic Acids Res., 25:4835-4841 (1997); Horn et al., Nucleic Acids Res., 25:4842-4849 (1997); Nilsen et al., J. Theor. Biol., 187:273-284 (1997).

[00189] The Invader™ assay is another technique for detecting single nucleotide variations that can be used for molecular profiling according to the invention. The Invader™ assay uses a novel linear signal amplification technology that improves upon the long turnaround times required of the typical PCR DNA sequenced-based analysis. See Cooksey et al., Antimicrobial Agents and Chemotherapy 44:1296-1301 (2000). This assay is based

on cleavage of a unique secondary structure formed between two overlapping oligonucleotides that hybridize to the target sequence of interest to form a "flap." Each "flap" then generates thousands of signals per hour. Thus, the results of this technique can be easily read, and the methods do not require exponential amplification of the DNA target. The Invader™ system utilizes two short DNA probes, which are hybridized to a DNA target. The structure formed by the hybridization event is recognized by a special cleavase enzyme that cuts one of the probes to release a short DNA "flap." Each released "flap" then binds to a fluorescently-labeled probe to form another cleavage structure. When the cleavase enzyme cuts the labeled probe, the probe emits a detectable fluorescence signal. See e.g. Lyamichev et al., Nat. Biotechnol., 17:292-296 (1999).

[00190] The rolling circle method is another method that avoids exponential amplification. Lizardi et al., Nature Genetics, 19:225-232 (1998) (which is incorporated herein by reference). For example, Sniper™, a commercial embodiment of this method, is a sensitive, high-throughput SNP scoring system designed for the accurate fluorescent detection of specific variants. For each nucleotide variant, two linear, allele-specific probes are designed. The two allele-specific probes are identical with the exception of the 3'-base, which is varied to complement the variant site. In the first stage of the assay, target DNA is denatured and then hybridized with a pair of single, allele-specific, open-circle oligonucleotide probes. When the 3'-base exactly complements the target DNA, ligation of the probe will preferentially occur. Subsequent detection of the circularized oligonucleotide probes is by rolling circle amplification, whereupon the amplified probe products are detected by fluorescence. See Clark and Pickering, Life Science News 6, 2000, Amersham Pharmacia Biotech (2000).

[00191] A number of other techniques that avoid amplification all together include, e.g., surface-enhanced resonance Raman scattering (SERRS), fluorescence correlation spectroscopy, and single-molecule electrophoresis. In SERRS, a chromophore-nucleic acid conjugate is absorbed onto colloidal silver and is irradiated with laser light at a resonant frequency of the chromophore. See Graham et al., Anal. Chem., 69:4703-4707 (1997). The fluorescence correlation spectroscopy is based on the spatio-temporal correlations among fluctuating light signals and trapping single molecules in an electric field. See Eigen et al., Proc. Natl. Acad. Sci. USA, 91:5740-5747 (1994). In single-molecule electrophoresis, the electrophoretic velocity of a fluorescently tagged nucleic acid is determined by measuring the time required for the molecule to travel a predetermined distance between two laser beams. See Castro et al., Anal. Chem., 67:3181-3186 (1995).

[00192] In addition, the allele-specific oligonucleotides (ASO) can also be used in in situ hybridization using tissues or cells as samples. The oligonucleotide probes which can hybridize differentially with the wild-type gene sequence or the gene sequence harboring a mutation may be labeled with radioactive isotopes, fluorescence, or other detectable markers. In situ hybridization techniques are well known in the art and their adaptation to the present invention for detecting the presence or absence of a nucleotide variant in the one or more gene of a particular individual should be apparent to a skilled artisan apprised of this disclosure.

[00193] Accordingly, the presence or absence of one or more genes nucleotide variant or amino acid variant in an individual can be determined using any of the detection methods described above.

[00194] Typically, once the presence or absence of one or more gene nucleotide variants or amino acid variants is determined, physicians or genetic counselors or patients or other researchers may be informed of the result. Specifically the result can be cast in a transmittable form that can be communicated or transmitted to other researchers or physicians or genetic counselors or patients. Such a form can vary and can be tangible or intangible. The result with regard to the presence or absence of a nucleotide variant of the present invention in

the individual tested can be embodied in descriptive statements, diagrams, photographs, charts, images or any other visual forms. For example, images of gel electrophoresis of PCR products can be used in explaining the results. Diagrams showing where a variant occurs in an individual's gene are also useful in indicating the testing results. The statements and visual forms can be recorded on a tangible media such as papers, computer readable media such as floppy disks, compact disks, etc., or on an intangible media, e.g., an electronic media in the form of email or website on internet or intranet. In addition, the result with regard to the presence or absence of a nucleotide variant or amino acid variant in the individual tested can also be recorded in a sound form and transmitted through any suitable media, e.g., analog or digital cable lines, fiber optic cables, etc., via telephone, facsimile, wireless mobile phone, internet phone and the like.

[00195] Thus, the information and data on a test result can be produced anywhere in the world and transmitted to a different location. For example, when a genotyping assay is conducted offshore, the information and data on a test result may be generated and cast in a transmittable form as described above. The test result in a transmittable form thus can be imported into the U.S. Accordingly, the present invention also encompasses a method for producing a transmittable form of information on the genotype of the two or more suspected cancer samples from an individual. The method comprises the steps of (1) determining the genotype of the DNA from the samples according to methods of the present invention; and (2) embodying the result of the determining step in a transmittable form. The transmittable form is the product of the production method.

[00196] ***In Situ Hybridization***

[00197] In situ hybridization assays are well known and are generally described in Angerer et al., Methods Enzymol. 152:649-660 (1987). In an in situ hybridization assay, cells, e.g., from a biopsy, are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters. FISH (fluorescence in situ hybridization) uses fluorescent probes that bind to only those parts of a sequence with which they show a high degree of sequence similarity.

[00198] In situ hybridization can be used to detect specific gene sequences in tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. Fluorescent in situ hybridization (FISH) uses a fluorescent probe to increase the sensitivity of in situ hybridization.

[00199] FISH is a cytogenetic technique used to detect and localize specific polynucleotide sequences in cells. For example, FISH can be used to detect DNA sequences on chromosomes. FISH can also be used to detect and localize specific RNAs, e.g., mRNAs, within tissue samples. In FISH uses fluorescent probes that bind to specific nucleotide sequences to which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out whether and where the fluorescent probes are bound. In addition to detecting specific nucleotide sequences, e.g., translocations, fusion, breaks, duplications and other chromosomal abnormalities, FISH can help define the spatial-temporal patterns of specific gene copy number and/or gene expression within cells and tissues.

[00200] Various types of FISH probes can be used to detect chromosome translocations. Dual color, single fusion probes can be useful in detecting cells possessing a specific chromosomal translocation. The DNA probe hybridization targets are located on one side of each of the two genetic breakpoints. “Extra signal” probes can reduce the frequency of normal cells exhibiting an abnormal FISH pattern due to the random co-localization of

probe signals in a normal nucleus. One large probe spans one breakpoint, while the other probe flanks the breakpoint on the other gene. Dual color, break apart probes are useful in cases where there may be multiple translocation partners associated with a known genetic breakpoint. This labeling scheme features two differently colored probes that hybridize to targets on opposite sides of a breakpoint in one gene. Dual color, dual fusion probes can reduce the number of normal nuclei exhibiting abnormal signal patterns. The probe offers advantages in detecting low levels of nuclei possessing a simple balanced translocation. Large probes span two breakpoints on different chromosomes. Such probes are available as Vysis probes from Abbott Laboratories, Abbott Park, IL.

[00201] Comparative Genomic Hybridization (CGH) comprises a molecular cytogenetic method of screening tumor samples for genetic changes showing characteristic patterns for copy number changes at chromosomal and subchromosomal levels. Alterations in patterns can be classified as DNA gains and losses. CGH employs the kinetics of in situ hybridization to compare the copy numbers of different DNA or RNA sequences from a sample, or the copy numbers of different DNA or RNA sequences in one sample to the copy numbers of the substantially identical sequences in another sample. In many useful applications of CGH, the DNA or RNA is isolated from a subject cell or cell population. The comparisons can be qualitative or quantitative. Procedures are described that permit determination of the absolute copy numbers of DNA sequences throughout the genome of a cell or cell population if the absolute copy number is known or determined for one or several sequences. The different sequences are discriminated from each other by the different locations of their binding sites when hybridized to a reference genome, usually metaphase chromosomes but in certain cases interphase nuclei. The copy number information originates from comparisons of the intensities of the hybridization signals among the different locations on the reference genome. The methods, techniques and applications of CGH are known, such as described in U.S. Pat. No. 6,335,167, and in U.S. App. Ser. No. 60/804,818, the relevant parts of which are herein incorporated by reference.

[00202] In an embodiment, CGH used to compare nucleic acids between diseased and healthy tissues. The method comprises isolating DNA from disease tissues (e.g., tumors) and reference tissues (e.g., healthy tissue) and labeling each with a different "color" or fluor. The two samples are mixed and hybridized to normal metaphase chromosomes. In the case of array or matrix CGH, the hybridization mixing is done on a slide with thousands of DNA probes. A variety of detection system can be used that basically determine the color ratio along the chromosomes to determine DNA regions that might be gained or lost in the diseased samples as compared to the reference.

[00203] Data and Analysis

[00204] The practice of the present invention may also employ conventional biology methods, software and systems. Computer software products of the invention typically include computer readable medium having computer-executable instructions for performing the logic steps of the method of the invention. Suitable computer readable medium include floppy disk, CD-ROM/DVD/DVD-ROM, hard-disk drive, flash memory, ROM/RAM, magnetic tapes and etc. The computer executable instructions may be written in a suitable computer language or combination of several languages. Basic computational biology methods are described in, for example Setubal and Meidanis et al., Introduction to Computational Biology Methods (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), Computational Methods in Molecular Biology, (Elsevier, Amsterdam, 1998); Rashidi and Buehler, Bioinformatics Basics: Application in Biological Science

and Medicine (CRC Press, London, 2000) and Ouelette and Bzevanis Bioinformatics: A Practical Guide for Analysis of Gene and Proteins (Wiley & Sons, Inc., 2.sup.nd ed., 2001). See U.S. Pat. No. 6,420,108.

[00205] The present invention may also make use of various computer program products and software for a variety of purposes, such as probe design, management of data, analysis, and instrument operation. See, U.S. Pat. Nos. 5,593,839, 5,795,716, 5,733,729, 5,974,164, 6,066,454, 6,090,555, 6,185,561, 6,188,783, 6,223,127, 6,229,911 and 6,308,170.

[00206] Additionally, the present invention relates to embodiments that include methods for providing genetic information over networks such as the Internet as shown in U.S. Ser. Nos. 10/197,621, 10/063,559 (U.S. Publication Number 20020183936), 10/065,856, 10/065,868, 10/328,818, 10/328,872, 10/423,403, and 60/482,389. For example, one or more molecular profiling techniques can be performed in one location, e.g., a city, state, country or continent, and the results can be transmitted to a different city, state, country or continent. Treatment selection can then be made in whole or in part in the second location. The methods of the invention comprise transmittal of information between different locations.

[00207] Molecular Profiling for Treatment Selection

[00208] The methods of the invention provide a candidate treatment selection for a subject in need thereof. Molecular profiling can be used to identify one or more candidate therapeutic agents for an individual suffering from a condition in which one or more of the biomarkers disclosed herein are targets for treatment. For example, the method can identify one or more chemotherapy treatments for a cancer. In an aspect, the invention provides a method comprising: performing an immunohistochemistry (IHC) analysis on a sample from the subject to determine an IHC expression profile on at least five proteins; performing a microarray analysis on the sample to determine a microarray expression profile on at least ten genes; performing a fluorescent in-situ hybridization (FISH) analysis on the sample to determine a FISH mutation profile on at least one gene; performing DNA sequencing on the sample to determine a sequencing mutation profile on at least one gene; and comparing the IHC expression profile, microarray expression profile, FISH mutation profile and sequencing mutation profile against a rules database, wherein the rules database comprises a mapping of treatments whose biological activity is known against diseased cells that: i) overexpress or underexpress one or more proteins included in the IHC expression profile; ii) overexpress or underexpress one or more genes included in the microarray expression profile; iii) have zero or more mutations in one or more genes included in the FISH mutation profile; and/or iv) have zero or more mutations in one or more genes included in the sequencing mutation profile; and identifying the treatment if the comparison against the rules database indicates that the treatment should have biological activity against the diseased cells; and the comparison against the rules database does not contraindicate the treatment for treating the diseased cells. The disease can be a cancer. The molecular profiling steps can be performed in any order. In some embodiments, not all of the molecular profiling steps are performed. As a non-limiting example, microarray analysis is not performed if the sample quality does not meet a threshold value, as described herein. In another example, sequencing is performed only if FISH analysis meets a threshold value. Any relevant biomarker can be assessed using one or more of the molecular profiling techniques described herein or known in the art. The marker need only have some direct or indirect association with a treatment to be useful.

[00209] Molecular profiling comprises the profiling of at least one gene (or gene product) for each assay technique that is performed. Different numbers of genes can be assayed with different techniques. Any marker

disclosed herein that is associated directly or indirectly with a target therapeutic can be assessed. For example, any "druggable target" comprising a target that can be modulated with a therapeutic agent such as a small molecule, is a candidate for inclusion in the molecular profiling methods of the invention. The molecular profiling can be based on either the gene, e.g., DNA sequence, and/or gene product, e.g., mRNA or protein. Such nucleic acid and/or polypeptide can be profiled as applicable as to presence or absence, level or amount, activity, mutation, sequence, haplotype, rearrangement, copy number, or other measurable characteristic. In some embodiments, a single gene and/or one or more corresponding gene products is assayed by more than one molecular profiling technique. A gene or gene product (also referred to herein as "marker" or "biomarker"), e.g., an mRNA or protein, is assessed using applicable techniques (e.g., to assess DNA, RNA, protein), including without limitation FISH, microarray, IHC, sequencing or immunoassay. Therefore, any of the markers disclosed herein can be assayed by a single molecular profiling technique or by multiple methods disclosed herein (e.g., a single marker is profiled by one or more of IHC, FISH, sequencing, microarray, etc.). In some embodiments, at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or at least about 100 genes or gene products are profiled by at least one technique, a plurality of techniques, or using a combination of FISH, microarray, IHC, and sequencing. In some embodiments, at least about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, 20,000, 21,000, 22,000, 23,000, 24,000, 25,000, 26,000, 27,000, 28,000, 29,000, 30,000, 31,000, 32,000, 33,000, 34,000, 35,000, 36,000, 37,000, 38,000, 39,000, 40,000, 41,000, 42,000, 43,000, 44,000, 45,000, 46,000, 47,000, 48,000, 49,000, or at least 50,000 genes or gene products are profiled using various techniques. The number of markers assayed can depend on the technique used. For example, microarray and massively parallel sequencing lend themselves to high throughput analysis. Because molecular profiling queries molecular characteristics of the tumor itself, this approach provides information on therapies that might not otherwise be considered based on the lineage of the tumor.

[00210] In some embodiments, a sample from a subject in need thereof is profiled using methods which include but are not limited to IHC expression profiling, microarray expression profiling, FISH mutation profiling, and/or sequencing mutation profiling (such as by PCR, RT-PCR, pyrosequencing) for one or more of the following: ABCC1, ABCG2, ACE2, ADA, ADH1C, ADH4, AGT, AR, AREG, ASNS, BCL2, BCRP, BDCA1, beta III tubulin, BIRC5, B-RAF, BRCA1, BRCA2, CA2, caveolin, CD20, CD25, CD33, CD52, CDA, CDKN2A, CDKN1A, CDKN1B, CDK2, CDW52, CES2, CK 14, CK 17, CK 5/6, c-KIT, c-Met, c-Myc, COX-2, Cyclin D1, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, E-Cadherin, ECGF1, EGFR, EML4-ALK fusion, EPHA2, Epiregulin, ER, ERBR2, ERCC1, ERCC3, EREG, ESR1, FLT1, folate receptor, FOLR1, FOLR2, FSHB, FSHPRH1, FSHR, FYN, GART, GNRH1, GNRHR1, GSTP1, HCK, HDAC1, hENT-1, Her2/Neu, HGF, HIF1A, HIG1, HSP90, HSP90AA1, HSPCA, IGF-1R, IGFRBP, IGFRBP3, IGFRBP4, IGFRBP5, IL13RA1, IL2RA, KDR, Ki67, KIT, K-RAS, LCK, LTB, Lymphotoxin Beta Receptor, LYN, MET, MGMT, MLH1, MMR, MRP1, MS4A1, MSH2, MSH5, Myc, NFKB1, NFKB2, NFKBIA, ODC1, OGFR, p16, p21, p27, p53, p95, PARP-1, PDGFC, PDGFR, PDGFRA, PDGFRB, PGP, PGR, PI3K, POLA, POLA1, PPARG, PPARGC1, PR, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Survivin, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TS, TXN, TXNRD1, TYMS, VDR, VEGF, VEGFA, VEGFC, VHL, YES1, ZAP70.

[00211] **Table 1** provides a listing of gene and corresponding protein symbols and names of many of the molecular profiling targets that are analyzed according to the methods of the invention. As understood by those of skill in the art, genes and proteins have developed a number of alternative names in the scientific literature. Thus, the listing in **Table 1** comprises an illustrative but not exhaustive compilation. A further listing of gene aliases and descriptions can be found using a variety of online databases, including GeneCards® (www.genecards.org), HUGO Gene Nomenclature (www.genenames.org), Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene), UniProtKB/Swiss-Prot (www.uniprot.org), UniProtKB/TrEMBL (www.uniprot.org), OMIM (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), GeneLoc (genecards.weizmann.ac.il/geneloc/), and Ensembl (www.ensembl.org). Generally, gene symbols and names below correspond to those approved by HUGO, and protein names are those recommended by UniProtKB/Swiss-Prot. Common alternatives are provided as well. Where a protein name indicates a precursor, the mature protein is also implied. Throughout the application, gene and protein symbols may be used interchangeably and the meaning can be derived from context, e.g., FISH is used to analyze nucleic acids whereas IHC is used to analyze protein.

Table 1: Gene and Protein Names

Gene Symbol	Gene Name	Protein Symbol	Protein Name
ABCB1, PGP	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1, MDR1, PGP	Multidrug resistance protein 1; P-glycoprotein
ABCC1, MRP1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	MRP1, ABCC1	Multidrug resistance-associated protein 1
ABCG2, BCRP	ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	ATP-binding cassette sub-family G member 2
ACE2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	ACE2	Angiotensin-converting enzyme 2 precursor
ADA	adenosine deaminase	ADA	Adenosine deaminase
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	ADH1G	Alcohol dehydrogenase 1C
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	ADH4	Alcohol dehydrogenase 4
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	ANGT, AGT	Angiotensinogen precursor
ALK	anaplastic lymphoma receptor tyrosine kinase	ALK	ALK tyrosine kinase receptor precursor
AR	androgen receptor	AR	Androgen receptor
AREG	amphiregulin	AREG	Amphiregulin precursor
ASNS	asparagine synthetase	ASNS	Asparagine synthetase [glutamine-hydrolyzing]
BCL2	B-cell CLL/lymphoma 2	BCL2	Apoptosis regulator Bcl-2
BDCA1, CD1C	CD1c molecule	CD1C	T-cell surface glycoprotein CD1c precursor
BIRC5	baculoviral IAP repeat-containing 5	BIRC5, Survivin	Baculoviral IAP repeat-containing protein 5; Survivin
BRAF	v-raf murine sarcoma viral oncogene homolog B1	B-RAF, BRAF	Serine/threonine-protein kinase B-raf
BRCA1	breast cancer 1, early onset	BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	breast cancer 2, early onset	BRCA2	Breast cancer type 2 susceptibility protein
CA2	carbonic anhydrase II	CA2	Carbonic anhydrase 2
CAV1	caveolin 1, caveolae protein, 22kDa	CAV1	Caveolin-1
CCND1	cyclin D1	CCND1, Cyclin D1, BCL-1	G1/S-specific cyclin-D1

CD20, MS4A1	membrane-spanning 4-domains, subfamily A, member 1	CD20	B-lymphocyte antigen CD20
CD25, IL2RA	interleukin 2 receptor, alpha	CD25	Interleukin-2 receptor subunit alpha precursor
CD33	CD33 molecule	CD33	Myeloid cell surface antigen CD33 precursor
CD52, CDW52	CD52 molecule	CD52	CAMPATH-1 antigen precursor
CDA	cytidine deaminase	CDA	Cytidine deaminase
CDH1, ECAD	cadherin 1, type 1, E-cadherin (epithelial)	E-Cad	Cadherin-1 precursor (E-cadherin)
CDK2	cyclin-dependent kinase 2	CDK2	Cell division protein kinase 2
CDKN1A, P21	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A, p21	Cyclin-dependent kinase inhibitor 1
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B, p27	Cyclin-dependent kinase inhibitor 1B
CDKN2A, P16	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	CD21A, p16	Cyclin-dependent kinase inhibitor 2A, isoforms 1/2/3
CES2	carboxylesterase 2 (intestine, liver)	CES2, EST2	Carboxylesterase 2 precursor
CK 5/6	cytokeratin 5 / cytokeratin 6	CK 5/6	Keratin, type II cytoskeletal 5; Keratin, type II cytoskeletal 6
CK14, KRT14	keratin 14	CK14	Keratin, type I cytoskeletal 14
CK17, KRT17	keratin 17	CK17	Keratin, type I cytoskeletal 17
COX2, PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	COX-2, PTGS2	Prostaglandin G/H synthase 2 precursor
DCK	deoxycytidine kinase	DCK	Deoxycytidine kinase
DHFR	dihydrofolate reductase	DHFR	Dihydrofolate reductase
DNMT1	DNA (cytosine-5-)-methyltransferase 1	DNMT1	DNA (cytosine-5)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	DNMT3A	DNA (cytosine-5)-methyltransferase 3A
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	DNMT3B	DNA (cytosine-5)-methyltransferase 3B
ECGF1, TYMP	thymidine phosphorylase	TYMP, PD-ECGF, ECDF1	Thymidine phosphorylase precursor
EGFR, ERBB1, HER1	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR, ERBB1, HER1	Epidermal growth factor receptor precursor
EML4	echinoderm microtubule associated protein like 4	EML4	Echinoderm microtubule-associated protein-like 4
EPHA2	EPH receptor A2	EPHA2	Ephrin type-A receptor 2 precursor
ER, ESR1	estrogen receptor 1	ER, ESR1	Estrogen receptor
ERBB2, HER2/NEU	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2, HER2, HER-2/neu	Receptor tyrosine-protein kinase erbB-2 precursor
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	ERCC1	DNA excision repair protein ERCC-1
ERCC3	excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	ERCC3	TFIIH basal transcription factor complex helicase XPB subunit
EREG	Epiregulin	EREG	Proepiregulin precursor

FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT-1, VEGFR1	Vascular endothelial growth factor receptor 1 precursor
FOLR1	folate receptor 1 (adult)	FOLR1	Folate receptor alpha precursor
FOLR2	folate receptor 2 (fetal)	FOLR2	Folate receptor beta precursor
FSHB	follicle stimulating hormone, beta polypeptide	FSHB	Follitropin subunit beta precursor
FSHPRH1, CENP1	centromere protein I	FSHPRH1, CENP1	Centromere protein I
FSHR	follicle stimulating hormone receptor	FSHR	Follicle-stimulating hormone receptor precursor
FYN	FYN oncogene related to SRC, FGR, YES	FYN	Tyrosine-protein kinase Fyn
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	GART, PUR2	Trifunctional purine biosynthetic protein adenosine-3
GNRH1	gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	GNRH1, GON1	Progonadoliberin-1 precursor
GNRHR1, GNRHR	gonadotropin-releasing hormone receptor	GNRHR1	Gonadotropin-releasing hormone receptor
GSTP1	glutathione S-transferase pi 1	GSTP1	Glutathione S-transferase P
HCK	hemopoietic cell kinase	HCK	Tyrosine-protein kinase HCK
HDAC1	histone deacetylase 1	HDAC1	Histone deacetylase 1
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	Hepatocyte growth factor precursor
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	HIF1A	Hypoxia-inducible factor 1-alpha
HIG1, HIGD1A, HIG1A	HIG1 hypoxia inducible domain family, member 1A	HIG1, HIGD1A, HIG1A	HIG1 domain family member 1A
HSP90AA1, HSP90, HSPCA	heat shock protein 90kDa alpha (cytosolic), class A member 1	HSP90, HSP90A	Heat shock protein HSP 90-alpha
IGF1R	insulin-like growth factor 1 receptor	IGF-1R	Insulin-like growth factor 1 receptor precursor
IGFBP3, IGFRBP3	insulin-like growth factor binding protein 3	IGFBP-3, IBP-3	Insulin-like growth factor-binding protein 3 precursor
IGFBP4, IGFRBP4	insulin-like growth factor binding protein 4	IGFBP-4, IBP-4	Insulin-like growth factor-binding protein 4 precursor
IGFBP5, IGFRBP5	insulin-like growth factor binding protein 5	IGFBP-5, IBP-5	Insulin-like growth factor-binding protein 5 precursor
IL13RA1	interleukin 13 receptor, alpha 1	IL-13RA1	Interleukin-13 receptor subunit alpha-1 precursor
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR, VEGFR2	Vascular endothelial growth factor receptor 2 precursor
KIT, c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT, c-KIT	Mast/stem cell growth factor receptor precursor
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	K-RAS	GTPase KRas precursor
LCK	lymphocyte-specific protein tyrosine kinase	LCK	Tyrosine-protein kinase Lck
LTB	lymphotoxin beta (TNF superfamily, member 3)	LTB, TNF3	Lymphotoxin-beta
LTBR	lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR, LTBR3, TNFR	Tumor necrosis factor receptor superfamily member 3 precursor

LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	Tyrosine-protein kinase Lyn
MET, c-MET	met proto-oncogene (hepatocyte growth factor receptor)	MET, c-MET	Hepatocyte growth factor receptor precursor
MGMT	O-6-methylguanine-DNA methyltransferase	MGMT	Methylated-DNA--protein-cysteine methyltransferase
MKI67, KI67	antigen identified by monoclonal antibody Ki-67	Ki67, Ki-67	Antigen KI-67
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	MLH1	DNA mismatch repair protein Mlh1
MMR	mismatch repair (refers to MLH1, MSH2, MSH5)		
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	MSH2	DNA mismatch repair protein Msh2
MSH5	mutS homolog 5 (E. coli)	MSH5, hMSH5	MutS protein homolog 5
MYC, c-MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC, c-MYC	Myc proto-oncogene protein
NBN, P95	nibrin	NBN, p95	Nibrin
NDGR1	N-myc downstream regulated 1	NDGR1	Protein NDGR1
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	Nuclear factor NF-kappa-B p105 subunit
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	Nuclear factor NF-kappa-B p100 subunit
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NF-kappa-B inhibitor alpha
ODC1	ornithine decarboxylase 1	ODC	Ornithine decarboxylase
OGFR	opioid growth factor receptor	OGFR	Opioid growth factor receptor
PARP1	poly (ADP-ribose) polymerase 1	PARP-1	Poly [ADP-ribose] polymerase 1
PDGFC	platelet derived growth factor C	PDGF-C, VEGF-E	Platelet-derived growth factor C precursor
PDGFR	platelet-derived growth factor receptor	PDGFR	Platelet-derived growth factor receptor
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	PDGFRA, PDGFR2, CD140 A	Alpha-type platelet-derived growth factor receptor precursor
PDGFRB	platelet-derived growth factor receptor, beta polypeptide	PDGFRB, PDGFR, PDGFR1, CD140 B	Beta-type platelet-derived growth factor receptor precursor
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	PI3K subunit p110α	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PSMD9, P27	proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	p27	26S proteasome non-ATPase regulatory subunit 9
PTEN	phosphatase and tensin homolog		
RRM1	ribonucleotide reductase M1	RRM1, RR1	Ribonucleoside-diphosphate reductase large subunit
RRM2	ribonucleotide reductase M2	RRM2, RR2M, RR2	Ribonucleoside-diphosphate reductase subunit M2
RRM2B	ribonucleotide reductase M2 B (TP53 inducible)	RRM2B, P53R2	Ribonucleoside-diphosphate reductase subunit M2 B
RXRB	retinoid X receptor, beta	RXRB	Retinoic acid receptor RXR-beta
RXRG	retinoid X receptor, gamma	RXRG, RXRC	Retinoic acid receptor RXR-gamma
SLC29A1	solute carrier family 29 (nucleoside transporters), member 1	ENT-1	Equilibrative nucleoside transporter 1
SPARC	secreted protein, acidic, cysteine-rich	SPARC	SPARC precursor; Osteonectin

	(osteonectin)		
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	SRC	Proto-oncogene tyrosine-protein kinase Src
SSTR1	somatostatin receptor 1	SSTR1, SSR1, SS1R	Somatostatin receptor type 1
SSTR2	somatostatin receptor 2	SSTR2, SSR2, SS2R	Somatostatin receptor type 2
SSTR3	somatostatin receptor 3	SSTR3, SSR3, SS3R	Somatostatin receptor type 3
SSTR4	somatostatin receptor 4	SSTR4, SSR4, SS4R	Somatostatin receptor type 4
SSTR5	somatostatin receptor 5	SSTR5, SSR5, SS5R	Somatostatin receptor type 5
TK1	thymidine kinase 1, soluble	TK1, KITH	Thymidine kinase, cytosolic
TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	TLE3	Transducin-like enhancer protein 3
TNF	tumor necrosis factor (TNF superfamily, member 2)	TNF, TNF-alpha, TNF-a	Tumor necrosis factor precursor
TOP1, TOPO1	topoisomerase (DNA) I	TOP1, TOPO1	DNA topoisomerase 1
TOP2A, TOPO2A	topoisomerase (DNA) II alpha 170kDa	TOP2A, TOP2, TOPO2A	DNA topoisomerase 2-alpha; Topoisomerase II alpha
TOP2B, TOPO2B	topoisomerase (DNA) II beta 180kDa	TOP2B, TOPO2B	DNA topoisomerase 2-beta; Topoisomerase II beta
TP53	tumor protein p53	p53	Cellular tumor antigen p53
TUBB3	tubulin, beta 3	Beta III tubulin, TUBB3, TUBB4	Tubulin beta-3 chain
TXN	thioredoxin	TXN, TRX, TRX-1	Thioredoxin
TXNRD1	thioredoxin reductase 1	TXNRD1, TXNR	Thioredoxin reductase 1, cytoplasmic; Oxidoreductase
TYMS, TS	thymidylate synthetase	TYMS, TS	Thymidylate synthase
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	Vitamin D3 receptor
VEGFA, VEGF	vascular endothelial growth factor A	VEGF-A, VEGF	Vascular endothelial growth factor A precursor
VEGFC	vascular endothelial growth factor C	VEGF-C	Vascular endothelial growth factor C precursor
VHL	von Hippel-Lindau tumor suppressor	VHL	Von Hippel-Lindau disease tumor suppressor
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	YES1, Yes, p61-Yes	Proto-oncogene tyrosine-protein kinase Yes
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa	ZAP-70	Tyrosine-protein kinase ZAP-70

[00212] In some embodiments, additional molecular profiling methods are performed. These can include without limitation PCR, RT-PCR, Q-PCR, SAGE, MPSS, immunoassays and other techniques to assess biological systems described herein or known to those of skill in the art. The choice of genes and gene products to be assayed can be updated over time as new treatments and new drug targets are identified. Once the expression or mutation of a biomarker is correlated with a treatment option, it can be assessed by molecular profiling. One of skill will appreciate that such molecular profiling is not limited to those techniques disclosed herein but comprises any methodology conventional for assessing nucleic acid or protein levels, sequence

information, or both. The methods of the invention can also take advantage of any improvements to current methods or new molecular profiling techniques developed in the future. In some embodiments, a gene or gene product is assessed by a single molecular profiling technique. In other embodiments, a gene and/or gene product is assessed by multiple molecular profiling techniques. In a non-limiting example, a gene sequence can be assayed by one or more of FISH and pyrosequencing analysis, the mRNA gene product can be assayed by one or more of RT-PCR and microarray, and the protein gene product can be assayed by one or more of IHC and immunoassay. One of skill will appreciate that any combination of biomarkers and molecular profiling techniques that will benefit disease treatment are contemplated by the invention.

[00213] The gene products used for IHC expression profiling include without limitation one or more of AR, BCRP, CD52, c-kit, ER, ERCC1, Her2/neu, MGMT, MRP1, PDGFR, PGP, PR, PTEN, RRM1, SPARC, TOP2A, TOPO1, and TS. In some embodiments, IHC analysis includes one or more of c-Met, EML4-ALK fusion, hENT-1, IGF-1R, MMR, p16, p21, p27, PARP-1, PI3K, and TLE3. IHC profiling of EGFR can also be performed. IHC is also used to detect or test for various gene products, including without limitation one or more of the following: EGFR, SPARC, C-kit, ER, PR, Androgen receptor, PGP, RRM1, TOPO1, BRCP1, MRP1, MGMT, PDGFR, DCK, ERCC1, Thymidylate synthase, Her2/neu, or TOPO2A. In some embodiments, IHC is used to detect on or more of the following proteins, including without limitation: ADA, AR, ASNA, BCL2, BRCA2, c-Met, CD33, CDW52, CES2, DNMT1, EGFR, EML4-ALK fusion, ERBB2, ERCC3, ESR1, FOLR2, GART, GSTP1, HDAC1, hENT-1, HIF1A, HSPCA, IGF-1R, IL2RA, KIT, MLH1, MMR, MS4A1, MASH2, NFKB2, NFKBIA, OGFR, p16, p21, p27, PARP-1, PI3K, PDGFC, PDGFRA, PDGFRB, PGR, POLA, PTEN, PTGS2, RAF1, RARA, RXRB, SPARC, SSTR1, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGF, VHL, or ZAP70. The proteins can be detected by IHC using monoclonal or polyclonal antibodies. In some embodiments, both are used. As an illustrative example, SPARC can be detected by anti-SPARC monoclonal (SPARC mono, SPARC m) and/or anti-SPARC polyclonal (SPARC poly, SPARC p) antibodies.

[00214] In some embodiments, IHC analysis according to the methods of the invention includes one or more of AR, c-Kit, CAV-1, CK 5/6, CK14, CK17, ECAD, ER, Her2/Neu, Ki67, MRP1, P53, PDGFR, PGP, PR, PTEN, SPARC, TLE3 and TS. All of these genes can be examined. As indicated by initial results of IHC or other molecular profiling methods as described herein, additional IHC assayscan be performed. In one embodiment, the additional IHC comprises that of p95, or p95, Cyclin D1 and EGFR. IHC can also be performed on IGFRBP3, IGFRBP4, IGFRBP5, or other forms of IGFRBP (e.g., IGFRBP1, IGFRBP2, IGFRBP6, IGFRBP7). In another embodiment, the additional IHC comprises that of one or more of BCRP, ERCC1, MGMT, P95, RRM1, TOP2A, and TOP1. In still another embodiment, the additional IHC comprises that of one or more of BCRP, Cyclin D1, EGFR, ERCC1, MGMT, P95, RRM1, TOP2A, and TOP1. All of these additional genes can be examined. The additional IHC can be selected on the basis of molecular characteristics of the tumor so that IHC is only performed where it is likely to indicate a candidate therapy for treating the cancer. As described herein, the molecular characteristics of the tumor determined can be determined by IHC combined with one or more of FISH, DNA microarray and mutation analysis.

[00215] Microarray expression profiling can be used to simultaneously measure the expression of one or more genes or gene products, including without limitation ABCC1, ABCG2, ADA, ALK, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EML4, EPHA2, ERBB2, ERCC1, ERCC3, ESR1, FLT1, FOLR2, FYN, GART, GNRH1, GSTP1, HCK,

HDAC1, hENT-1, HIF1A, HSP90AA1, IGF-1R, IL2RA, HSP90AA1, KDR, KIT, LCK, LYN, MET, MGMT, MLH1, MMR, MS4A1, MSH2, NFKB1, NFKB2, OGFR, PDGFC, PDGFRA, PDGFRB, p16, p21, p27, PARP-1, PGR, PI3K, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70. In some embodiments, the genes used for the microarray expression profiling comprise one or more of: EGFR, SPARC, C-kit, ER, PR, Androgen receptor, PGP, RRM1, TOPO1, BRCP1, MRP1, MGMT, PDGFR, DCK, ERCC1, Thymidylate synthase, Her2/neu, TOPO2A, ADA, AR, ASNA, BCL2, BRCA2, CD33, CDW52, CES2, DNMT1, EGFR, ERBB2, ERCC3, ESR1, FOLR2, GART, GSTP1, HDAC1, HIF1A, HSPCA, IL2RA, KIT, MLH1, MS4A1, MASH2, NFKB2, NFKBIA, OGFR, PDGFC, PDGFRA, PDGFRB, PGR, POLA, PTEN, PTGS2, RAF1, RARA, RXRB, SPARC, SSTR1, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGF, VHL, and ZAP70. The microarray expression profiling can be performed using a low density microarray, an expression microarray, a comparative genomic hybridization (CGH) microarray, a single nucleotide polymorphism (SNP) microarray, a proteomic array an antibody array, or other array as disclosed herein or known to those of skill in the art. In some embodiments, high throughput expression arrays are used. Such systems include without limitation commercially available systems from Agilent or Illumina, as described in more detail herein.

[00216] Microarray expression profiling can be used to simultaneously measure the expression of one or more genes or gene products, including without limitation ABCC1, ABCG2, ADA, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EPHA2, ERBB2, ERCC1, ERCC3, ESR1, FLT1, FOLR2, FYN, GART, GNRH1, GSTP1, HCK, HDAC1, HIF1A, HSP90AA1, IL2RA, KDR, KIT, LCK, LYN, MGMT, MLH1, MS4A1, MSH2, NFKB1, NFKB2, OGFR, PDGFC, PDGFRA, PDGFRB, PGR, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70.

[00217] FISH mutation profiling can be used to profile one or more of EGFR and HER2. In some embodiments, FISH is used to detect or test for one or more of the following genes, including without limitation: EGFR, SPARC, C-kit, ER, PR, AR, PGP, RRM1, TOPO1, BRCP1, MRP1, MGMT, PDGFR, DCK, ERCC1, TS, HER2, or TOPO2A. In some embodiments, FISH is used to detect or test for one or more of EML4-ALK fusion and IGF-1R. In some embodiments, FISH is used to detect or test various biomarkers, including without limitation one or more of the following: ADA, AR, ASNA, BCL2, BRCA2, c-Met, CD33, CDW52, CES2, DNMT1, EGFR, EML4-ALK fusion, ERBB2, ERCC3, ESR1, FOLR2, GART, GSTP1, HDAC1, hENT-1, HIF1A, HSPCA, IGF-1R, IL2RA, KIT, MLH1, MMR, MS4A1, MASH2, NFKB2, NFKBIA, OGFR, p16, p21, p27, PARP-1, PI3K, PDGFC, PDGFRA, PDGFRB, PGR, POLA, PTEN, PTGS2, RAF1, RARA, RXRB, SPARC, SSTR1, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGF, VHL, or ZAP70.

[00218] In some embodiments, FISH is used to detect or test for HER2. Depending on the results of the HER2 analysis and other molecular profiling techniques, additional FISH testing may be performed. The additional FISH testing can comprise that of CMYC and/or TOP2A. For example, FISH testing may indicate that a cancer is HER2+. The cancer may be a breast cancer. HER2+ cancers may then be followed up by FISH testing for CMYC and TOP2A, whereas HER2- cancers are followed up with FISH testing for CMYC. For some cancers,

e.g., triple negative breast cancer (i.e., ER-/PR-/HER2-), additional FISH testing may not be performed. The decision whether to perform additional FISH testing can be guided by whether the additional FISH testing is likely to reveal information about candidate therapies for the cancer. The additional FISH can be selected on the basis of molecular characteristics of the tumor so that FISH is only performed where it is likely to indicate a candidate therapy for treating the cancer. As described herein, the molecular characteristics of the tumor determined can be determined by one or more of IHC, FISH, DNA microarray and sequence analysis.

[00219] In some embodiments, the genes used for the mutation profiling comprise one or more of KRAS, BRAF, c-KIT and EGFR. Mutation profiling can be determined by sequencing, including Sanger sequencing, array sequencing, pyrosequencing, NextGen sequencing, etc. Sequence analysis may reveal that genes harbor activating mutations so that drugs that inhibit activity are indicated for treatment. Alternately, sequence analysis may reveal that genes harbor mutations that inhibit or eliminate activity, thereby indicating treatment for compensating therapies. In embodiments, sequence analysis comprises that of exon 9 and 11 of c-KIT. Sequencing may also be performed on EGFR-kinase domain exons 18, 19, 20, and 21. Mutations, amplifications or misregulations of EGFR or its family members are implicated in about 30% of all epithelial cancers. Sequencing can also be performed on PI3K, encoded by the PIK3CA gene. This gene is a found mutated in many cancers. Sequencing analysis can also comprise assessing mutations in one or more ABCC1, ABCG2, ADA, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, c-Met, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EPHA2, EML4-ALK fusion, ERBB2, ERCC1, ERCC3, ESR1, FLT1, FOLR2, FYN, GART, GNRH1, GSTP1, HCK, HDAC1, hENT-1, HIF1A, HSP90AA1, IL2RA, HSP90AA1, KDR, KIT, LCK, LYN, MGMT, MLH1, MMR, MS4A1, MSH2, NFKB1, NFKB2, OGFR, p16, p21, p27, PARP-1, PI3K, PDGFC, PDGFRA, PDGFRB, PGR, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70.

[00220] In some embodiments, mutational analysis is performed on PIK3CA. The decision whether to perform mutational analysis on PIK3CA can be guided by whether this testing is likely to reveal information about candidate therapies for the cancer. The PIK3CA mutational analysis can be selected on the basis of molecular characteristics of the tumor so that the analysis is only performed where it is likely to indicate a candidate therapy for treating the cancer. As described herein, the molecular characteristics of the tumor determined can be determined by one or more of IHC, FISH, DNA microarray and sequence analysis. In one embodiment, PIK3CA is analyzed for a HER2+ cancer. The cancer can be a breast cancer.

[00221] In a related aspect, the invention provides a method of identifying a candidate treatment for a subject in need thereof by using molecular profiling of sets of known biomarkers. For example, the method can identify a chemotherapeutic agent for an individual with a cancer. The method comprises: obtaining a sample from the subject; performing an immunohistochemistry (IHC) analysis on the sample to determine an IHC expression profile on one or more, e.g. 2, 3, 4, 5, 6,7, 8, 9, 10 or more, of: SPARC, PGP, Her2/neu, ER, PR, c-kit, AR, CD52, PDGFR, TOP2A, TS, ERCC1, RRM1, BCRP, TOPO1, PTEN, MGMT, MRP1, c-Met, EML4-ALK fusion, hENT-1, IGF-1R, MMR, p16, p21, p27, PARP-1, PI3K, and TLE3; performing a microarray analysis on the sample to determine a microarray expression profile on one or more, e.g. 2, 3, 4, 5, 6,7, 8, 9, 10 or more, of: ABCC1, ABCG2, ADA, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EPHA2, ERBB2, ERCC1, ERCC3, ESR1, FLT1,

FOLR2, FYN, GART, GNRH1, GSTP1, HCK, HDAC1, HIF1A, HSP90AA1, IGF-1R, IL2RA, HSP90AA1, KDR, KIT, LCK, LYN, MGMT, MLH1, MS4A1, MSH2, NFKB1, NFKB2, OGFR, PARP1, PDGFC, PDGFRA, PDGFRB, PGR, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70; performing a fluorescent in-situ hybridization (FISH) analysis on the sample to determine a FISH mutation profile on at least one of EGFR, HER2, EML4-ALK fusion and IGF-1R; performing DNA sequencing on the sample to determine a sequencing mutation profile on at least one of KRAS, BRAF, c-KIT, PI3K (*PIK3CA*) and EGFR; and comparing the IHC expression profile, microarray expression profile, FISH mutation profile and sequencing mutation profile against a rules database, wherein the rules database comprises a mapping of treatments whose biological activity is known against diseased cells that: i) overexpress or underexpress one or more proteins included in the IHC expression profile; ii) overexpress or underexpress one or more genes included in the microarray expression profile; iii) have zero or more mutations in one or more genes included in the FISH mutation profile; and/or iv) have zero or more mutations in one or more genes included in the sequencing mutation profile; and identifying the treatment if the comparison against the rules database indicates that the treatment should have biological activity against the disease; and the comparison against the rules database does not contraindicate the treatment for treating the disease. The disease can be a cancer. The molecular profiling steps can be performed in any order. In some embodiments, not all of the molecular profiling steps are performed. As a non-limiting example, microarray analysis is not performed if the sample quality does not meet a threshold value, as described herein. In some embodiments, the IHC expression profiling is performed on at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the gene products above. In some embodiments, the microarray expression profiling is performed on at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the genes listed above.

[00222] In a related aspect, the invention provides a method of identifying a candidate treatment for a subject in need thereof by using molecular profiling of defined sets of known biomarkers. For example, the method can identify a chemotherapeutic agent for an individual with a cancer. The method comprises: obtaining a sample from the subject, wherein the sample comprises formalin-fixed paraffin-embedded (FFPE) tissue or fresh frozen tissue, and wherein the sample comprises cancer cells; performing an immunohistochemistry (IHC) analysis on the sample to determine an IHC expression profile on at least: SPARC, PGP, Her2/neu, ER, PR, c-kit, AR, CD52, PDGFR, TOP2A, TS, ERCC1, RRM1, BCRP, TOPO1, PTEN, MGMT, MRP1, c-Met, EML4-ALK fusion, hENT-1, IGF-1R, MMR, p16, p21, p27, PARP-1, PI3K, and TLE3; performing a microarray analysis on the sample to determine a microarray expression profile on at least: ABCC1, ABCG2, ADA, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EPHA2, ERBB2, ERCC1, ERCC3, ESR1, FLT1, FOLR2, FYN, GART, GNRH1, GSTP1, HCK, HDAC1, HIF1A, HSP90AA1, IGF-1R, IL2RA, HSP90AA1, KDR, KIT, LCK, LYN, MGMT, MLH1, MS4A1, MSH2, NFKB1, NFKB2, OGFR, PARP1, PDGFC, PDGFRA, PDGFRB, PGR, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70; performing a fluorescent in-situ hybridization (FISH) analysis on the sample to determine a FISH mutation profile on at least one of EGFR, HER2, EML4-ALK fusion and IGF-1R; performing DNA sequencing on the sample to determine a sequencing mutation profile on at least KRAS, BRAF, c-KIT, PI3K (*PIK3CA*) and EGFR. The IHC

expression profile, microarray expression profile, FISH mutation profile and sequencing mutation profile are compared against a rules database, wherein the rules database comprises a mapping of treatments whose biological activity is known against diseased cells that: i) overexpress or underexpress one or more proteins included in the IHC expression profile; ii) overexpress or underexpress one or more genes included in the microarray expression profile; iii) have zero or more mutations in one or more genes included in the FISH mutation profile; or iv) have zero or more mutations in one or more genes included in the sequencing mutation profile; and identifying the treatment if the comparison against the rules database indicates that the treatment should have biological activity against the disease; and the comparison against the rules database does not contraindicate the treatment for treating the disease. The disease can be a cancer. The molecular profiling steps can be performed in any order. In some embodiments, not all of the molecular profiling steps are performed. As a non-limiting example, microarray analysis is not performed if the sample quality does not meet a threshold value, as described herein. In some embodiments, the biological material is mRNA and the quality control test comprises a A260/A280 ratio and/or a Ct value of RT-PCR using a housekeeping gene, e.g., RPL13a. In embodiments, the mRNA does not pass the quality control test if the A260/A280 ratio < 1.5 or the RPL13a Ct value is > 30. In that case, microarray analysis may not be performed. Alternately, microarray results may be attenuated, e.g., given a lower priority as compared to the results of other molecular profiling techniques.

[00223] In some embodiments, molecular profiling is always performed on certain genes or gene products, whereas the profiling of other genes or gene products is optional. For example, IHC expression profiling may be performed on at least SPARC, TOP2A and/or PTEN. Similarly, microarray expression profiling may be performed on at least CD52. In other embodiments, genes in addition to those listed above are used to identify a treatment. For example, the group of genes used for the IHC expression profiling can further comprise DCK, EGFR, BRCA1, CK 14, CK 17, CK 5/6, E-Cadherin, p95, PARP-1, SPARC and TLE3. In some embodiments, the group of genes used for the IHC expression profiling further comprises Cox-2 and/or Ki-67. In some embodiments, HSPCA is assayed by microarray analysis. In some embodiments, FISH mutation is performed on c-Myc and TOP2A. In some embodiments, sequencing is performed on PI3K.

[00224] The methods of the invention can be used in any setting wherein differential expression or mutation analysis have been linked to efficacy of various treatments. In some embodiments, the methods are used to identify candidate treatments for a subject having a cancer. Under these conditions, the sample used for molecular profiling preferably comprises cancer cells. The percentage of cancer in a sample can be determined by methods known to those of skill in the art, e.g., using pathology techniques. Cancer cells can also be enriched from a sample, e.g., using microdissection techniques or the like. A sample may be required to have a certain threshold of cancer cells before it is used for molecular profiling. The threshold can be at least about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 95% cancer cells. The threshold can depend on the analysis method. For example, a technique that reveals expression in individual cells may require a lower threshold that a technique that used a sample extracted from a mixture of different cells. In some embodiments, the diseased sample is compared to a normal sample taken from the same patient, e.g., adjacent but non-cancer tissue.

[00225] In embodiments, the methods of the invention are used detect gene fusions, such as those listed in International PCT Patent Application PCT/US2010/000407, filed February 11, 2010, which application is incorporated by reference in its entirety herein. See **Table 2** therein. A fusion gene is a hybrid gene created by the juxtaposition of two previously separate genesThis can occur by chromosomal translocation or inversion,

deletion or via trans-splicing. The resulting fusion gene can cause abnormal temporal and spatial expression of genes, leading to abnormal expression of cell growth factors, angiogenesis factors, tumor promoters or other factors contributing to the neoplastic transformation of the cell and the creation of a tumor. For example, such fusion genes can be oncogenic due to the juxtaposition of: 1) a strong promoter region of one gene next to the coding region of a cell growth factor, tumor promoter or other gene promoting oncogenesis leading to elevated gene expression, or 2) due to the fusion of coding regions of two different genes, giving rise to a chimeric gene and thus a chimeric protein with abnormal activity. Fusion genes are characteristic of many cancers. Once a therapeutic intervention is associated with a fusion, the presence of that fusion in any type of cancer identifies the therapeutic intervention as a candidate therapy for treating the cancer.

[00226] The presence of fusion genes can be used to guide therapeutic selection. For example, the BCR-ABL gene fusion is a characteristic molecular aberration in ~90% of chronic myelogenous leukemia (CML) and in a subset of acute leukemias (Kurzrock *et al., Annals of Internal Medicine* 2003; **138**:819-830). The BCR-ABL results from a translocation between chromosomes 9 and 22, commonly referred to as the Philadelphia chromosome or Philadelphia translocation. The translocation brings together the 5' region of the BCR gene and the 3' region of ABL1, generating a chimeric BCR-ABL1 gene, which encodes a protein with constitutively active tyrosine kinase activity (Mittleman et al., Nature *Reviews Cancer* 2007; 7:233-245). The aberrant tyrosine kinase activity leads to de-regulated cell signaling, cell growth and cell survival, apoptosis resistance and growth factor independence, all of which contribute to the pathophysiology of leukemia (Kurzrock et al., *Annals of Internal Medicine* 2003; **138**:819-830). Patients with the Philadelphia chromosome are treated with imatinib and other targeted therapies. Imatinib binds to the site of the constitutive tyrosine kinase activity of the fusion protein and prevents its activity. Imatinib treatment has led to molecular responses (disappearance of BCR-ABL+ blood cells) and improved progression-free survival in BCR-ABL+ CML patients (Kantarjian *et al., Clinical Cancer Research* 2007; **13**:1089-1097).

[00227] Another fusion gene, IGH-MYC, is a defining feature of ~80% of Burkitt's lymphoma (Ferry et al. *Oncologist* 2006; 11:375-83). The causal event for this is a translocation between chromosomes 8 and 14, bringing the c-Myc oncogene adjacent to the strong promoter of the immunoglobulin heavy chain gene, causing *c-myc* overexpression (Mittleman et al., *Nature Reviews Cancer* 2007; 7:233-245). The c-*myc* rearrangement is a pivotal event in lymphomagenesis as it results in a perpetually proliferative state. It has wide ranging effects on progression through the cell cycle, cellular differentiation, apoptosis, and cell adhesion (Ferry et al. *Oncologist* 2006; 11:375-83).

[00228] A number of recurrent fusion genes have been catalogued in the Mittleman database (cgap.nci.nih.gov/Chromosomes/Mitelman). The gene fusions described therein can be used to characterize neoplasms and cancers and guide therapy using the subject methods described herein. For example, TMPRSS2-ERG, TMPRSS2-ETV and SLC45A3-ELK4 fusions can be detected to characterize prostate cancer; and ETV6-NTRK3 and ODZ4-NRG1 can be used to characterize breast cancer. The EML4-ALK, RLF-MYCL1, TGF-ALK, or CD74-ROS1 fusions can be used to characterize a lung cancer. The ACSL3-ETV1, C15ORF21-ETV1, FLJ35294-ETV1, HERV-ETV1, TMPRSS2-ERG, TMPRSS2-ETV1/4/5, TMPRSS2-ETV4/5, SLC5A3-ERG, SLC5A3-ETV1, SLC5A3-ETV5 or KLK2-ETV4 fusions can be used to characterize a prostate cancer. The GOPC-ROS1 fusion can be used to characterize a brain cancer. The CHCHD7-PLAG1, CTNNB1-PLAG1, FHIT-HMGA2, HMGA2-NFIB, LIFR-PLAG1, or TCEA1-PLAG1 fusions can be used to characterize a head

and neck cancer. The ALPHA-TFEB, NONO-TFE3, PRCC-TFE3, SFPQ-TFE3, CLTC-TFE3, or MALAT1-TFEB fusions can be used to characterize a renal cell carcinoma (RCC). The AKAP9-BRAF, CCDC6-RET, ERC1-RETM, GOLGA5-RET, HOOK3-RET, HRH4-RET, KTN1-RET, NCOA4-RET, PCM1-RET, PRKARA1A-RET, RFG-RET, RFG9-RET, Ria-RET, TGF-NTRK1, TPM3-NTRK1, TPM3-TPR, TPR-MET, TPR-NTRK1, TRIM24-RET, TRIM27-RET or TRIM33-RET fusions can be used to characterize a thyroid cancer and/or papillary thyroid carcinoma; and the PAX8-PPARy fusion can be analyzed to characterize a follicular thyroid cancer. Fusions that are associated with hematological malignancies include without limitation TTL-ETV6, CDK6-MLL, CDK6-TLX3, ETV6-FLT3, ETV6-RUNX1, ETV6-TTL, MLL-AFF1, MLL-AFF3, MLL-AFF4, MLL-GAS7, TCBA1-ETV6, TCF3-PBX1 or TCF3-TFPT, which are characteristic of acute lymphocytic leukemia (ALL); BCL11B-TLX3, IL2-TNFRFS17, NUP214-ABL1, NUP98-CCDC28A, TAL1-STIL, or ETV6-ABL2, which are characteristic of T-cell acute lymphocytic leukemia (T-ALL); ATIC-ALK, KIAA1618-ALK, MSN-ALK, MYH9-ALK, NPM1-ALK, TGF-ALK or TPM3-ALK, which are characteristic of anaplastic large cell lymphoma *(*ALCL); BCR-ABL1, BCR-JAK2, ETV6-EVI1, ETV6-MN1 or ETV6-TCBA1, characteristic of chronic myelogenous leukemia (CML); CBFB-MYH11, CHIC2-ETV6, ETV6-ABL1, ETV6-ABL2, ETV6-ARNT, ETV6-CDX2, ETV6-HLXB9, ETV6-PER1, MEF2D-DAZAP1, AML-AFF1, MLL-ARHGAP26, MLL-ARHGEF12, MLL-CASC5, MLL-CBL,MLL-CREBBP, MLL-DAB21P, MLL-ELL, MLL-EP300, MLL-EPS15, MLL-FNBP1, MLL-FOXO3A, MLL-GMPS, MLL-GPHN, MLL-MLLT1, MLL-MLLT11, MLL-MLLT3, MLL-MLLT6, MLL-MYO1F, MLL-PICALM, MLL-SEPT2, MLL-SEPT6, MLL-SORBS2, MYST3-SORBS2, MYST-CREBBP, NPM1-MLF1, NUP98-HOXA13, PRDM16-EVI1, RABEP1-PDGFRB, RUNX1-EVI1, RUNX1-MDS1, RUNX1-RPL22, RUNX1-RUNX1T1, RUNX1-SH3D19, RUNX1-USP42, RUNX1-YTHDF2, RUNX1-ZNF687, or TAF15-ZNF-384, which are characteristic of acute myeloid leukemia (AML); CCND1-FSTL3, which is characteristic of chronic lymphocytic leukemia (CLL); BCL3-MYC, MYC-BTG1, BCL7A-MYC, BRWD3-ARHGAP20 or BTG1-MYC, which are characteristic of B-cell chronic lymphocytic leukemia (B-CLL); CITTA-BCL6, CLTC-ALK, IL21R-BCL6, PIM1-BCL6, TFCR-BCL6, IKZF1-BCL6 or SEC31A-ALK, which are characteristic of diffuse large B-cell lymphomas (DLBCL); FLIP1-PDGFRA, FLT3-ETV6, KIAA1509-PDGFRA, PDE4DIP-PDGFRB, NIN-PDGFRB, TP53BP1-PDGFRB, or TPM3-PDGFRB, which are characteristic of hyper eosinophilia / chronic eosinophilia; and IGH-MYC or LCP1-BCL6, which are characteristic of Burkitt's lymphoma. One of skill will understand that additional fusions, including those yet to be identified to date, can be used to guide treatment once their presence is associated with a therapeutic intervention.

[00229] The fusion genes and gene products can be detected using one or more techniques described herein. In some embodiments, the sequence of the gene or corresponding mRNA is determined, e.g., using Sanger sequencing, NextGen sequencing, pyrosequencing, DNA microarrays, etc. Chromosomal abnormalities can be assessed using FISH or PCR techniques, among others. For example, a break apart probe can be used for FISH detection of ALK fusions such as *EML4-ALK*, *KIF5B-ALK* and/or *TFG-ALK*. As an alternate, PCR can be used to amplify the fusion product, wherein amplification or lack thereof indicates the presence or absence of the fusion, respectively. In some embodiments, the fusion protein fusion is detected. Appropriate methods for protein analysis include without limitation mass spectroscopy, electrophoresis (e.g., 2D gel electrophoresis or SDS-PAGE) or antibody related techniques, including immunoassay, protein array or immunohistochemistry.

The techniques can be combined. As a non-limiting example, indication of an ALK fusion by FISH can be confirmed for ALK expression using IHC, or vice versa.

[00230] Treatment Selection

[00231] The systems and methods allow identification of one or more therapeutic targets whose projected efficacy can be linked to therapeutic efficacy, ultimately based on the molecular profiling. Illustrative schemes for using molecular profiling to identify a treatment regime are shown in **FIGs. 1** and **39**, each of which is described in further detail herein. The invention comprises use of molecular profiling results to suggest associations with treatment responses. In an embodiment, the appropriate biomarkers for molecular profiling are selected on the basis of the subject's tumor type. These suggested biomarkers can be used to modify a default list of biomarkers. In other embodiments, the molecular profiling is independent of the source material. In some embodiments, rules are used to provide the suggested chemotherapy treatments based on the molecular profiling test results. In an embodiment, the rules are generated from abstracts of the peer reviewed clinical oncology literature. Expert opinion rules can be used but are optional. In an embodiment, clinical citations are assessed for their relevance to the methods of the invention using a hierarchy derived from the evidence grading system used by the United States Preventive Services Taskforce. The "best evidence" can be used as the basis for a rule. The simplest rules are constructed in the format of "if biomarker positive then treatment option one, else treatment option two." Treatment options comprise no treatment with a specific drug, treatment with a specific drug or treatment with a combination of drugs. In some embodiments, more complex rules are constructed that involve the interaction of two or more biomarkers. In such cases, the more complex interactions are typically supported by clinical studies that analyze the interaction between the biomarkers included in the rule. Finally, a report can be generated that describes the association of the chemotherapy response and the biomarker and a summary statement of the best evidence supporting the treatments selected. Ultimately, the treating physician will decide on the best course of treatment.

[00232] As a non-limiting example, molecular profiling might reveal that the EGFR gene is amplified or overexpressed, thus indicating selection of a treatment that can block EGFR activity, such as the monoclonal antibody inhibitors cetuximab and panitumumab, or small molecule kinase inhibitors effective in patients with activating mutations in EGFR such as gefitinib, erlotinib, and lapatinib. Other anti-EGFR monoclonal antibodies in clinical development include zalutumumab, nimotuzumab, and matuzumab. The candidate treatment selected can depend on the setting revealed by molecular profiling. For example, kinase inhibitors are often prescribed with EGFR is found to have activating mutations. Continuing with the illustrative embodiment, molecular profiling may also reveal that some or all of these treatments are likely to be less effective. For example, patients taking gefitinib or erlotinib eventually develop drug resistance mutations in EGFR. Accordingly, the presence of a drug resistance mutation would contraindicate selection of the small molecule kinase inhibitors. One of skill will appreciate that this example can be expanded to guide the selection of other candidate treatments that act against genes or gene products whose differential expression is revealed by molecular profiling. Similarly, candidate agents known to be effective against diseased cells carrying certain nucleic acid variants can be selected if molecular profiling reveals such variants.

[00233] As another example, consider the drug imatinib, currently marketed by Novartis as Gleevec in the US in the form of imatinib mesylate. Imatinib is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of a number of tyrosine kinase enzymes. It occupies the tyrosine kinase active site, leading to a

decrease in kinase activity. Imatinib has been shown to block the activity of Abelson cytoplasmic tyrosine kinase (ABL), c-Kit and the platelet-derived growth factor receptor (PDGFR). Thus, imatinib can be indicated as a candidate therapeutic for a cancer determined by molecular profiling to overexpress ABL, c-KIT or PDGFR. Imatinib can be indicated as a candidate therapeutic for a cancer determined by molecular profiling to have mutations in ABL, c-KIT or PDGFR that alter their activity, e.g., constitutive kinase activity of ABLs caused by the BCR-ABL mutation. As an inhibitor of PDGFR, imatinib mesylate appears to have utility in the treatment of a variety of dermatological diseases.

[00234] Cancer therapies that can be identified as candidate treatments by the methods of the invention include without limitation: 13-cis-Retinoic Acid, 2-CdA, 2-Chlorodeoxyadenosine, 5-Azacitidine, 5-Fluorouracil, 5-FU, 6-Mercaptopurine, 6-MP, 6-TG, 6-Thioguanine, Abraxane, Accutane®, Actinomycin-D, Adriamycin®, Adrucil®, Afinitor®, Agrylin®, Ala-Cort®, Aldesleukin, Alemtuzumab, ALIMTA, Alitretinoin, Alkaban-AQ®, Alkeran®, All-transretinoic Acid, Alpha Interferon, Altretamine, Amethopterin, Amifostine, Aminoglutethimide, Anagrelide, Anandron®, Anastrozole, Arabinosylcytosine, Ara-C, Aranesp®, Aredia®, Arimidex®, Aromasin®, Arranon®, Arsenic Trioxide, Asparaginase, ATRA, Avastin®, Azacitidine, BCG, BCNU, Bendamustine, Bevacizumab, Bexarotene, BEXXAR®, Bicalutamide, BiCNU, Blenoxane®, Bleomycin, Bortezomib, Busulfan, Busulfex®, C225, Calcium Leucovorin, Campath®, Camptosar®, Camptothecin-11, Capecitabine, Carac™, Carboplatin, Carmustine, Carmustine Wafer, Casodex®, CC-5013, CCI-779, CCNU, CDDP, CeeNU, Cerubidine®, Cetuximab, Chlorambucil, Cisplatin, Citrovorum Factor, Cladribine, Cortisone, Cosmegen®, CPT-11, Cyclophosphamide, Cytadren®, Cytarabine, Cytarabine Liposomal, Cytosar-U®, Cytoxan®, Dacarbazine, Dacogen, Dactinomycin, Darbepoetin Alfa, Dasatinib, Daunomycin Daunorubicin, Daunorubicin Hydrochloride, Daunorubicin Liposomal, DaunoXome®, Decadron, Decitabine, Delta-Cortef®, Deltasone®, Denileukin, Diftitox, DepoCyt™, Dexamethasone, Dexamethasone Acetate Dexamethasone Sodium Phosphate, Dexasone, Dexrazoxane, DHAD, DIC, Diodex Docetaxel, Doxil®, Doxorubicin, Doxorubicin Liposomal, Droxia™, DTIC, DTIC-Dome®, Duralone®, Efudex®, Eligard™, Ellence™, Eloxatin™, Elspar®, Emcyt®, Epirubicin, Epoetin Alfa, Erbitux, Erlotinib, Erwinia L-asparaginase, Estramustine, Ethyol Etopophos®, Etoposide, Etoposide Phosphate, Eulexin®, Everolimus, Evista®, Exemestane, Fareston®, Faslodex®, Femara®, Filgrastim, Floxuridine, Fludara®, Fludarabine, Fluoroplex®, Fluorouracil, Fluorouracil (cream), Fluoxymesterone, Flutamide, Folinic Acid, FUDR®, Fulvestrant, G-CSF, Gefitinib, Gemcitabine, Gemtuzumab ozogamicin, Gemzar, Gleevec™, Gliadel® Wafer, GM-CSF, Goserelin, Granulocyte - Colony Stimulating Factor, Granulocyte Macrophage Colony Stimulating Factor, Halotestin®, Herceptin®, Hexadrol, Hexalen®, Hexamethylmelamine, HMM, Hycamtin®, Hydrea®, Hydrocort Acetate®, Hydrocortisone, Hydrocortisone Sodium Phosphate, Hydrocortisone Sodium Succinate, Hydrocortone Phosphate, Hydroxyurea, Ibritumomab, Ibritumomab, Tiuxetan, Idamycin®, Idarubicin, Ifex®, IFN-alpha, Ifosfamide, IL-11, IL-2, Imatinib mesylate, Imidazole Carboxamide, Interferon alfa, Interferon Alfa-2b (PEG Conjugate), Interleukin - 2, Interleukin-11, Intron A® (interferon alfa-2b), Iressa®, Irinotecan, Isotretinoin, Ixabepilone, Ixempra™, Kidrolase (t), Lanacort®, Lapatinib, L-asparaginase, LCR, Lenalidomide, Letrozole, Leucovorin, Leukeran, Leukine™, Leuprolide, Leurocristine, Leustatin™, Liposomal Ara-C Liquid Pred®, Lomustine, L-PAM, L-Sarcolysin, Lupron®, Lupron Depot®, Matulane®, Maxidex, Mechlorethamine, Mechlorethamine Hydrochloride, Medralone®, Medrol®, Megace®, Megestrol, Megestrol Acetate, Melphalan, Mercaptopurine, Mesna, Mesnex™, Methotrexate, Methotrexate Sodium, Methylprednisolone, Meticorten®,

Mitomycin, Mitomycin-C, Mitoxantrone, M-Prednisol®, MTC, MTX, Mustargen®, Mustine, Mutamycin®, Myleran®, Mylocel™, Mylotarg®, Navelbine®, Nelarabine, Neosar®, Neulasta™, Neumega®, Neupogen®, Nexavar®, Nilandron®, Nilutamide, Nipent®, Nitrogen Mustard, Novaldex®, Novantrone®, Octreotide, Octreotide acetate, Oncospar®, Oncovin®, Ontak®, Onxal™, Oprevelkin, Orapred®, Orasone®, Oxaliplatin, Paclitaxel, Paclitaxel Protein-bound, Pamidronate, Panitumumab, Panretin®, Paraplatin®, Pediapred®, PEG Interferon, Pegaspargase, Pegfilgrastim, PEG-INTRON™, PEG-L-asparaginase, PEMETREXED, Pentostatin, Phenylalanine Mustard, Platinol®, Platinol-AQ®, Prednisolone, Prednisone, Prelone®, Procarbazine, PROCRIT®, Proleukin®, Prolifeprospan 20 with Carmustine Implant, Purinethol®, Raloxifene, Revlimid®, Rheumatrex®, Rituxan®, Rituximab, Roferon-A® (Interferon Alfa-2a), Rubex®, Rubidomycin hydrochloride, Sandostatin®, Sandostatin LAR®, Sargramostim, Solu-Cortef®, Solu-Medrol®, Sorafenib, SPRYCEL™, STI-571, Streptozocin, SU11248, Sunitinib, Sutent®, Tamoxifen, Tarceva®, Targretin®, Taxol®, Taxotere®, Temodar®, Temozolomide, Temsirolimus, Teniposide, TESPA, Thalidomide, Thalomid®, TheraCys®, Thioguanine, Thioguanine Tabloid®, Thiophosphoamide, Thioplex®, Thiotepa, TICE®, Toposar®, Topotecan, Toremifene, Torisel®, Tositumomab, Trastuzumab, Treanda®, Tretinoin, Trexall™, Trisenox®, TSPA, TYKERB®, VCR, Vectibix™, Velban®, Velcade®, VePesid®, Vesanoid®, Viadur™, Vidaza®, Vinblastine, Vinblastine Sulfate, Vincasar Pfs®, Vincristine, Vinorelbine, Vinorelbine tartrate, VLB, VM-26, Vorinostat, VP-16, Vumon®, Xeloda®, Zanosar®, Zevalin™, Zinecard®, Zoladex®, Zoledronic acid, Zolinza, Zometa®, and any appropriate combinations thereof.

[00235] The candidate treatments identified according to the subject methods can be chosen from the class of therapeutic agents identified as Anthracyclines and related substances, Anti-androgens, Anti-estrogens, Antigrowth hormones (e.g., Somatostatin analogs), Combination therapy (e.g., vincristine, bcnu, melphalan, cyclophosphamide, prednisone (VBMCP)), DNA methyltransferase inhibitors, Endocrine therapy - Enzyme inhibitor, Endocrine therapy - other hormone antagonists and related agents, Folic acid analogs (e.g., methotrexate), Folic acid analogs (e.g., pemetrexed), Gonadotropin releasing hormone analogs, Gonadotropin-releasing hormones, Monoclonal antibodies (EGFR-Targeted - e.g., panitumumab, cetuximab), Monoclonal antibodies (Her2-Targeted - e.g., trastuzumab), Monoclonal antibodies (Multi-Targeted - e.g., alemtuzumab), Other alkylating agents, Other antineoplastic agents (e.g., asparaginase), Other antineoplastic agents (e.g., ATRA), Other antineoplastic agents (e.g., bexarotene), Other antineoplastic agents (e.g., celecoxib), Other antineoplastic agents (e.g., gemcitabine), Other antineoplastic agents (e.g., hydroxyurea), Other antineoplastic agents (e.g., irinotecan, topotecan), Other antineoplastic agents (e.g., pentostatin), Other cytotoxic antibiotics, Platinum compounds, Podophyllotoxin derivatives (e.g., etoposide), Progestogens, Protein kinase inhibitors (EGFR-Targeted), Protein kinase inhibitors (Her2 targeted therapy - e.g., lapatinib), Pyrimidine analogs (e.g., cytarabine), Pyrimidine analogs (e.g., fluoropyrimidines), Salicylic acid and derivatives (e.g., aspirin), Src-family protein tyrosine kinase inhibitors (e.g., dasatinib), Taxanes, Taxanes (e.g., nab-paclitaxel), Vinca Alkaloids and analogs, Vitamin D and analogs, Monoclonal antibodies (Multi-Targeted - e.g., bevacizumab), Protein kinase inhibitors (e.g., imatinib, sorafenib, sunitinib).

[00236] In some embodiments, the candidate treatments identified according to the subject methods are chosen from at least the groups of treatments consisting of 5-fluorouracil, abarelix, alemtuzumab, aminoglutethimide, anastrozole, asparaginase, aspirin, ATRA, azacitidine, bevacizumab, bexarotene, bicalutamide, calcitriol, capecitabine, carboplatin, celecoxib, cetuximab, chemotherapy, cholecalciferol, cisplatin, cytarabine, dasatinib,

daunorubicin, decitabine, doxorubicin, epirubicin, erlotinib, etoposide, exemestane, flutamide, fulvestrant, gefitinib, gemcitabine, gonadorelin, goserelin, hydroxyurea, imatinib, irinotecan, lapatinib, letrozole, leuprolide, liposomal-doxorubicin, medroxyprogesterone, megestrol, megestrol acetate, methotrexate, mitomycin, nab-paclitaxel, octreotide, oxaliplatin, paclitaxel, panitumumab, pegaspargase, pemetrexed, pentostatin, sorafenib, sunitinib, tamoxifen, Taxanes, temozolomide, toremifene, trastuzumab, VBMCP, and vincristine.

[00237] Rules Engine

[00238] In some embodiments, a database is created that maps treatments and molecular profiling results. The treatment information can include the projected efficacy of a therapeutic agent against cells having certain attributes that can be measured by molecular profiling. The molecular profiling can include differential expression or mutations in certain genes, proteins, or other biological molecules of interest. Through the mapping, the results of the molecular profiling can be compared against the database to select treatments. The database can include both positive and negative mappings between treatments and molecular profiling results. In some embodiments, the mapping is created by reviewing the literature for links between biological agents and therapeutic agents. For example, a journal article, patent publication or patent application publication, scientific presentation, etc can be reviewed for potential mappings. The mapping can include results of in vivo, e.g., animal studies or clinical trials, or in vitro experiments, e.g., cell culture. Any mappings that are found can be entered into the database, e.g., cytotoxic effects of a therapeutic agent against cells expressing a gene or protein. In this manner, the database can be continuously updated. It will be appreciated that the methods of the invention are updated as well.

[00239] The rules for the mappings can contain a variety of supplemental information. In some embodiments, the database contains prioritization criteria. For example, a treatment with more projected efficacy in a given setting can be preferred over a treatment projected to have lesser efficacy. A mapping derived from a certain setting, e.g., a clinical trial, may be prioritized over a mapping derived from another setting, e.g., cell culture experiments. A treatment with strong literature support may be prioritized over a treatment supported by more preliminary results. A treatment generally applied to the type of disease in question, e.g., cancer of a certain tissue origin, may be prioritized over a treatment that is not indicated for that particular disease. Mappings can include both positive and negative correlations between a treatment and a molecular profiling result. In a non-limiting example, one mapping might suggest use of a kinase inhibitor like erlotinib against a tumor having an activating mutation in EGFR, whereas another mapping might suggest against that treatment if the EGFR also has a drug resistance mutation. Similarly, a treatment might be indicated as effective in cells that overexpress a certain gene or protein but indicated as not effective if the gene or protein is underexpressed.

[00240] The selection of a candidate treatment for an individual can be based on molecular profiling results from any one or more of the methods described. Alternatively, selection of a candidate treatment for an individual can be based on molecular profiling results from more than one of the methods described. For example, selection of treatment for an individual can be based on molecular profiling results from FISH alone, IHC alone, or microarray analysis alone. In other embodiments, selection of treatment for an individual can be based on molecular profiling results from IHC, FISH, and microarray analysis; IHC and FISH; IHC and microarray analysis, or FISH and microarray analysis. Selection of treatment for an individual can also be based on molecular profiling results from sequencing or other methods of mutation detection. Molecular profiling results may include mutation analysis along with one or more methods, such as IHC, immunoassay, and/or

microarray analysis. Different combinations and sequential results can be used. For example, treatment can be prioritized according the results obtained by molecular profiling. In an embodiment, the prioritization is based on the following algorithm: 1) IHC/FISH and microarray indicates same target as a first priority; 2) IHC positive result alone next priority; or 3) microarray positive result alone as last priority. Sequencing can also be used to guide selection. In some embodiments, sequencing reveals a drug resistance mutation so that the effected drug is not selected even if techniques including IHC, microarray and/or FISH indicate differential expression of the target molecule. Any such contraindication, e.g., differential expression or mutation of another gene or gene product may override selection of a treatment.

[00241] An illustrative listing of microarray expression results versus predicted treatments is presented in **Table 2**. As disclosed herein, molecular profiling is performed to determine whether a gene or gene product is differentially expressed in a sample as compared to a control. The expression status of the gene or gene product is used to select agents that are predicted to be efficacious or not. For example, **Table 2** shows that overexpression of the ADA gene or protein points to pentostatin as a possible treatment. On the other hand, underexpression of the ADA gene or protein implicates resistance to cytarabine, suggesting that cytarabine is not an optimal treatment.

Table 2: Molecular Profiling Results and Predicted Treatments

Gene Name	Expression Status	Candidate Agent(s)	Possible Resistance
ADA	Overexpressed	pentostatin	
ADA	Underexpressed		cytarabine
AR	Overexpressed	abarelix, bicalutamide, flutamide, gonadorelin, goserelin, leuprolide	
ASNS	Underexpressed	asparaginase, pegaspargase	
BCRP (ABCG2)	Overexpressed		cisplatin, carboplatin, irinotecan, topotecan
BRCA1	Underexpressed	mitomycin	
BRCA2	Underexpressed	mitomycin	
CD52	Overexpressed	alemtuzumab	
CDA	Overexpressed		cytarabine
CES2	Overexpressed	irinotecan	
c-kit	Overexpressed	sorafenib, sunitinib, imatinib	
COX-2	Overexpressed	celecoxib	
DCK	Overexpressed	gemcitabine	cytarabine
DHFR	Underexpressed	methotrexate, pemetrexed	
DHFR	Overexpressed		methotrexate
DNMT1	Overexpressed	azacitidine, decitabine	
DNMT3A	Overexpressed	azacitidine, decitabine	
DNMT3B	Overexpressed	azacitidine, decitabine	
EGFR	Overexpressed	erlotinib, gefitinib, cetuximab, panitumumab	
EML4-ALK	Overexpressed (present)	crizotinib	
EPHA2	Overexpressed	dasatinib	
ER	Overexpressed	anastrazole, exemestane, fulvestrant, letrozole, megestrol, tamoxifen, medroxyprogesterone, toremifene, aminoglutethimide	
ERCC1	Overexpressed		carboplatin, cisplatin
GART	Underexpressed	pemetrexed	

HER-2 (ERBB2)	Overexpressed	trastuzumab, lapatinib	
HIF-1α	Overexpressed	sorafenib, sunitinib, bevacizumab	
IκB-α	Overexpressed	bortezomib	
MGMT	Underexpressed	temozolomide	
MGMT	Overexpressed		temozolomide
MRP1 (ABCC1)	Overexpressed		etoposide, paclitaxel, docetaxel, vinblastine, vinorelbine, topotecan, teniposide
P-gp (ABCB1)	Overexpressed		doxorubicin, etoposide, epirubicin, paclitaxel, docetaxel, vinblastine, vinorelbine, topotecan, teniposide, liposomal doxorubicin
PDGFR-α	Overexpressed	sorafenib, sunitinib, imatinib	
PDGFR-β	Overexpressed	sorafenib, sunitinib, imatinib	
PR	Overexpressed	exemestane, fulvestrant, gonadorelin, goserelin, medroxyprogesterone, megestrol, tamoxifen, toremifene	
RARA	Overexpressed	ATRA	
RRM1	Underexpressed	gemcitabine, hydroxyurea	
RRM2	Underexpressed	gemcitabine, hydroxyurea	
RRM2B	Underexpressed	gemcitabine, hydroxyurea	
RXR-α	Overexpressed	bexarotene	
RXR-β	Overexpressed	bexarotene	
SPARC	Overexpressed	nab-paclitaxel	
SRC	Overexpressed	dasatinib	
SSTR2	Overexpressed	octreotide	
SSTR5	Overexpressed	octreotide	
TOPO I	Overexpressed	irinotecan, topotecan	
TOPO IIα	Overexpressed	doxorubicin, epirubicin, liposomal- doxorubicin	
TOPO IIβ	Overexpressed	doxorubicin, epirubicin, liposomal- doxorubicin	
TS	Underexpressed	capecitabine, 5-fluorouracil, pemetrexed	
TS	Overexpressed		capecitabine, 5-fluorouracil
VDR	Overexpressed	calcitriol, cholecalciferol	
VEGFR1 (Flt1)	Overexpressed	sorafenib, sunitinib, bevacizumab	
VEGFR2	Overexpressed	sorafenib, sunitinib, bevacizumab	
VHL	Underexpressed	sorafenib, sunitinib	

[00242] **Table 3** presents an illustrative rules summary for treatment selection. The table is ordered by groups of related therapeutic agents. Each row describes a rule that maps the information derived from molecular profiling with an indication of benefit or lack of benefit for the therapeutic agent. Thus, the database contains a mapping of treatments whose biological activity is known against cancer cells that have alterations in certain genes or gene products, including gene copy alterations, chromosomal abnormalities, overexpression of or

underexpression of one or more genes or gene products, or have various mutations. For each agent, a Lineage is presented as applicable which corresponds to a type of cancer associated with use of the agent. Agents with Benefit are listed along with a Benefit Summary Statement that describes molecular profiling information that relates to the predicted beneficial agent. Similarly, agents with Lack of Benefit are listed along with a Lack of Benefit Summary Statement that describes molecular profiling information that relates to the lack of benefit associated with the agent. Finally, the molecular profiling Criteria are shown. In the criteria, results from analysis using DNA microarray (DMA), IHC, FISH, and mutation analysis (MA) for one or more biomarkers is listed. For microarray analysis, expression can be reported as over (overexpressed) or under (underexpressed). When these criteria are met according to the application of the molecular profiling techniques to a sample, then the therapeutic agent or agents are predicted to have a benefit or lack of benefit as indicated in the corresponding row.

Table 3: Rules Summary for Treatment Selection

Therapeutic Agent	**Agents with Benefit**	**Benefit Summary**	**Agents with Lack of Benefit**	**Lack of Benefit Summary**	**Criteria**
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC Over. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC Over. IHC: SPARC Monoclonal Negative. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC. IHC: SPARC Monoclonal Negative. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC Over. IHC: SPARC Monoclonal. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC. IHC: SPARC Monoclonal. IHC: SPARC Polyclonal Above Threshold
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC Over. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal Negative
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal Negative
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with			DMA: SPARC Over. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal

		benefit from nab-Paclitaxel.			
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC. IHC: SPARC Monoclonal Above Threshold. IHC: SPARC Polyclonal
Taxanes (nab-paclitaxel)	nab-paclitaxel	High expression of SPARC has been associated with benefit from nab-Paclitaxel.			DMA: SPARC Over. IHC: SPARC Monoclonal. IHC: SPARC Polyclonal

[00243] Further drug associations and rules that are used in embodiments of the invention are found in U.S. Provisional Patent Application 61/427,788, filed December 28, 2010; U.S. Patent Application 12/658,770, filed February 12, 2010; International PCT Patent Application PCT/US2010/000407, filed February 11, 2010; and International PCT Patent Application PCT/US2010/54366, filed October 27, 2010; all of which applications are incorporated by reference herein in their entirety. See e.g., "Table 4: Rules Summary for Treatment Selection" of PCT/US2010/54366 and "Table 4: Rules Summary for Treatment Selection" of U.S. 61/427,788.

[00244] The efficacy of various therapeutic agents given particular assay results, such as those in **Table 3** or the patent applications listed directly above, can be derived from reviewing, analyzing and rendering conclusions on empirical evidence, such as that is available the medical literature or other medical knowledge base. The results are used to guide the selection of certain therapeutic agents in a prioritized list for use in treatment of an individual. When molecular profiling results are obtained, e.g., differential expression or mutation of a gene or gene product, the results can be compared against the database to guide treatment selection. The set of rules in the database can be updated as new treatments and new treatment data become available. In some embodiments, the rules database is updated continuously. In some embodiments, the rules database is updated on a periodic basis. Any relevant correlative or comparative approach can be used to compare the molecular profiling results to the rules database. In one embodiment, a gene or gene product is identified as differentially expressed by molecular profiling. The rules database is queried to select entries for that gene or gene product. Treatment selection information selected from the rules database is extracted and used to select a treatment. The information, e.g., to recommend or not recommend a particular treatment, can be dependent on whether the gene or gene product is over or underexpressed, or has other abnormalities at the genetic or protein levels as compared to a reference. In some cases, multiple rules and treatments may be pulled from a database comprising the comprehensive rules set depending on the results of the molecular profiling. In some embodiments, the treatment options are presented in a prioritized list. In some embodiments, the treatment options are presented without prioritization information. In either case, an individual, e.g., the treating physician or similar caregiver may choose from the available options.

[00245] The methods described herein are used to prolong survival of a subject by providing personalized treatment. In some embodiments, the subject has been previously treated with one or more therapeutic agents to treat the disease, e.g., a cancer. The cancer may be refractory to one of these agents, e.g., by acquiring drug resistance mutations. In some embodiments, the cancer is metastatic. In some embodiments, the subject has not previously been treated with one or more therapeutic agents identified by the method. Using molecular profiling, candidate treatments can be selected regardless of the stage, anatomical location, or anatomical origin of the cancer cells.

[00246] Progression-free survival (PFS) denotes the chances of staying free of disease progression for an individual or a group of individuals suffering from a disease, e.g., a cancer, after initiating a course of treatment. It can refer to the percentage of individuals in a group whose disease is likely to remain stable (e.g., not show signs of progression) after a specified duration of time. Progression-free survival rates are an indication of the effectiveness of a particular treatment. Similarly, disease-free survival (DFS) denotes the chances of staying free of disease after initiating a particular treatment for an individual or a group of individuals suffering from a cancer. It can refer to the percentage of individuals in a group who are likely to be free of disease after a specified duration of time. Disease-free survival rates are an indication of the effectiveness of a particular treatment. Treatment strategies can be compared on the basis of the PFS or DFS that is achieved in similar groups of patients. Disease-free survival is often used with the term overall survival when cancer survival is described.

[00247] The candidate treatment selected by molecular profiling according to the invention can be compared to a non-molecular profiling selected treatment by comparing the progression free survival (PFS) using therapy selected by molecular profiling (period B) with PFS for the most recent therapy on which the patient has just progressed (period A). See **FIG. 8**. In one setting, a PFS(B)/PFS(A) ratio ≥ 1.3 was used to indicate that the molecular profiling selected therapy provides benefit for patient *(Robert Temple, Clinical measurement in drug evaluation. Edited by Wu Ningano and G.T. Thicker John Wiley and Sons Ltd. 1995; Von Hoff, D.D. Clin Can Res. 4: 1079, 1999: Dhani et al. Clin Cancer Res. 15: 118-123, 2009).* Other methods of comparing the treatment selected by molecular profiling to a non-molecular profiling selected treatment include determining response rate (RECIST) and percent of patients without progression or death at 4 months. The term "about" as used in the context of a numerical value for PFS means a variation of +/- ten percent (10%) relative to the numerical value. The PFS from a treatment selected by molecular profiling can be extended by at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or at least 90% as compared to a non-molecular profiling selected treatment. In some embodiments, the PFS from a treatment selected by molecular profiling can be extended by at least 100%, 150%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or at least about 1000% as compared to a non-molecular profiling selected treatment. In yet other embodiments, the PFS ratio (PFS on molecular profiling selected therapy or new treatment / PFS on prior therapy or treatment) is at least about 1.3. In yet other embodiments, the PFS ratio is at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0. In yet other embodiments, the PFS ratio is at least about 3, 4, 5, 6, 7, 8, 9 or 10.

[00248] Similarly, the DFS can be compared in patients whose treatment is selected with or without molecular profiling. In embodiments, DFS from a treatment selected by molecular profiling is extended by at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or at least 90% as compared to a non-molecular profiling selected treatment. In some embodiments, the DFS from a treatment selected by molecular profiling can be extended by at least 100%, 150%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or at least about 1000% as compared to a non-molecular profiling selected treatment. In yet other embodiments, the DFS ratio (DFS on molecular profiling selected therapy or new treatment / DFS on prior therapy or treatment) is at least about 1.3. In yet other embodiments, the DFS ratio is at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0. In yet other embodiments, the DFS ratio is at least about 3, 4, 5, 6, 7, 8, 9 or 10.

[00249] In some embodiments, the candidate treatment of the invention will not increase the PFS ratio or the DFS ratio in the patient, nevertheless molecular profiling provides invaluable patient benefit. For example, in

some instances no preferable treatment has been identified for the patient. In such cases, molecular profiling provides a method to identify a candidate treatment where none is currently identified. The molecular profiling may extend PFS, DFS or lifespan by at least 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 2 months, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 13 months, 14 months, 15 months, 16 months, 17 months, 18 months, 19 months, 20 months, 21 months, 22 months, 23 months, 24 months or 2 years. The molecular profiling may extend PFS, DFS or lifespan by at least 2 ½ years, 3 years, 4 years, 5 years, or more. In some embodiments, the methods of the invention improve outcome so that patient is in remission.

[00250] The effectiveness of a treatment can be monitored by other measures. A complete response (CR) comprises a complete disappearance of the disease: no disease is evident on examination, scans or other tests. A partial response (PR) refers to some disease remaining in the body, but there has been a decrease in size or number of the lesions by 30% or more. Stable disease (SD) refers to a disease that has remained relatively unchanged in size and number of lesions. Generally, less than a 50% decrease or a slight increase in size would be described as stable disease. Progressive disease (PD) means that the disease has increased in size or number on treatment. In some embodiments, molecular profiling according to the invention results in a complete response or partial response. In some embodiments, the methods of the invention result in stable disease. In some embodiments, the invention is able to achieve stable disease where non-molecular profiling results in progressive disease.

[00251] Molecular Profiling Methods

[00252] FIG. 1 shows a flowchart of an illustrative embodiment of a method 50 for determining individualized medical intervention for a particular disease state that utilizes molecular profiling of a patient's biological specimen that is non disease specific. In order to determine a medical intervention for a particular disease state using molecular profiling that is independent of disease lineage diagnosis (i.e. not single disease restricted), at least one test is performed for at least one target from a biological sample of a diseased patient in step 52. A target is defined as any molecular finding that may be obtained from molecular testing. For example, a target may include one or more genes, one or more gene expressed proteins, one or more molecular mechanisms, and/or combinations of such. For example, the expression level of a target can be determined by the analysis of mRNA levels or the target or gene, or protein levels of the gene. Tests for finding such targets may include, but are not limited, fluorescent in-situ hybridization (FISH), an in-situ hybridization (ISH), and other molecular tests known to those skilled in the art. PCR-based methods, such as real-time PCR or quantitative PCR can be used. Furthermore, microarray analysis, such as a comparative genomic hybridization (CGH) micro array, a single nucleotide polymorphism (SNP) microarray, a proteomic array, or antibody array analysis can also be used in the methods disclosed herein. In some embodiments, microarray analysis comprises identifying whether a gene is up-regulated or down-regulated relative to a reference with a significance of $p<0.001$. Tests or analyses of targets can also comprise immunohistochemical (IHC) analysis. In some embodiments, IHC analysis comprises determining whether 30% or more of a sample is stained, if the staining intensity is +2 or greater, or both.

[00253] Furthermore, the methods disclosed herein also including profiling more than one target. For example, the expression of a plurality of genes can be identified. Furthermore, identification of a plurality of targets in a sample can be by one method or by various means. For example, the expression of a first gene can be determined by one method and the expression level of a second gene determined by a different method.

Alternatively, the same method can be used to detect the expression level of the first and second gene. For example, the first method can be IHC and the second by microarray analysis, such as detecting the gene expression of a gene.

[00254] In some embodiments, molecular profiling can also including identifying a genetic variant, such as a mutation, polymorphism (such as a SNP), deletion, or insertion of a target. For example, identifying a SNP in a gene can be determined by microarray analysis, real-time PCR, or sequencing. Other methods disclosed herein can also be used to identify variants of one or more targets.

[00255] Accordingly, one or more of the following may be performed: an IHC analysis in step 54, a microanalysis in step 56, and other molecular tests know to those skilled in the art in step 58.

[00256] Biological samples are obtained from diseased patients by taking a biopsy of a tumor, conducting minimally invasive surgery if no recent tumor is available, obtaining a sample of the patient's blood, or a sample of any other biological fluid including, but not limited to, cell extracts, nuclear extracts, cell lysates or biological products or substances of biological origin such as excretions, blood, sera, plasma, urine, sputum, tears, feces, saliva, membrane extracts, and the like.

[00257] In step 60, a determination is made as to whether one or more of the targets that were tested for in step 52 exhibit a change in expression compared to a normal reference for that particular target. In one illustrative method of the invention, an IHC analysis may be performed in step 54 and a determination as to whether any targets from the IHC analysis exhibit a change in expression is made in step 64 by determining whether 30% or more of the biological sample cells were +2 or greater staining for the particular target. It will be understood by those skilled in the art that there will be instances where +1 or greater staining will indicate a change in expression in that staining results may vary depending on the technician performing the test and type of target being tested. In another illustrative embodiment of the invention, a micro array analysis may be performed in step 56 and a determination as to whether any targets from the micro array analysis exhibit a change in expression is made in step 66 by identifying which targets are up-regulated or down-regulated by determining whether the fold change in expression for a particular target relative to a normal tissue of origin reference is significant at $p< 0.001$. A change in expression may also be evidenced by an absence of one or more genes, gene expressed proteins, molecular mechanisms, or other molecular findings.

[00258] After determining which targets exhibit a change in expression in step 60, at least one non-disease specific agent is identified that interacts with each target having a changed expression in step 70. An agent may be any drug or compound having a therapeutic effect. A non-disease specific agent is a therapeutic drug or compound not previously associated with treating the patient's diagnosed disease that is capable of interacting with the target from the patient's biological sample that has exhibited a change in expression. Some of the non-disease specific agents that have been found to interact with specific targets found in different cancer patients are shown in **Table 4** below.

Table 4: Illustrative target-drug associations

Patients	**Target(s) Found**	**Treatment(s)**
Advanced Pancreatic Cancer	HER 2/*neu* (IHC/Array)	Herceptin™
Advanced Pancreatic Cancer	EGFR (IHC), HIF 1α	Erbitux™, Rapamycin™

Advanced Ovarian Cancer	ERCC3 (Array)	Irofulvene
Advanced Adenoid Cystic Carcinoma	Vitamin D receptors, Androgen receptors	Calcitriol™, Flutamide™

[00259] Molecular Profiling Targets

[00260] The present invention provides methods and systems for analyzing diseased tissue using molecular profiling as previously described above. Because the methods rely on analysis of the characteristics of the tumor under analysis, the methods can be applied in for any tumor or any stage of disease, such an advanced stage of disease or a metastatic tumor of unknown origin. As described herein, a tumor or cancer sample is analyzed for molecular characteristics in order to predict or identify a candidate therapeutic treatment. The molecular characteristics can include the expression of genes or gene products, assessment of gene copy number, or mutational analysis. Any relevant determinable characteristic that can assist in prediction or identification of a candidate therapeutic can be included within the methods of the invention.

[00261] The biomarker patterns or biomarker signature sets can be determined for tumor types, diseased tissue types, or diseased cells including without limitation adipose, adrenal cortex, adrenal gland, adrenal gland – medulla, appendix, bladder, blood vessel, bone, bone cartilage, brain, breast, cartilage, cervix, colon, colon sigmoid, dendritic cells, skeletal muscle, endometrium, esophagus, fallopian tube, fibroblast, gallbladder, kidney, larynx, liver, lung, lymph node, melanocytes, mesothelial lining, myoepithelial cells, osteoblasts, ovary, pancreas, parotid, prostate, salivary gland, sinus tissue, skeletal muscle, skin, small intestine, smooth muscle, stomach, synovium, joint lining tissue, tendon, testis, thymus, thyroid, uterus, and uterus corpus.

[00262] The methods of the present invention can be used for selecting a treatment of any cancer or tumor type, including but not limited to breast cancer (including HER2+ breast cancer, HER2- breast cancer, ER/PR+, HER2- breast cancer, or triple negative breast cancer), pancreatic cancer, cancer of the colon and/or rectum, leukemia, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer, brain cancer, cancer of the larynx, gallbladder, parathyroid, thyroid, adrenal, neural tissue, head and neck, stomach, bronchi, kidneys, basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, small-cell lung tumor, islet cell carcinoma, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuroma, intestinal ganglioneuroma, hyperplastic corneal nerve tumor, marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyoma, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis fungoides, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant hypercalcemia, renal cell tumor, polycythermia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas, malignant melanomas, and epidermoid carcinomas. The cancer or tumor can comprise, without limitation, a carcinoma, a sarcoma, a lymphoma or leukemia, a germ cell tumor, a blastoma, or other cancers. Carcinomas that can be assessed using the subject methods include without limitation epithelial neoplasms, squamous cell neoplasms, squamous cell carcinoma, basal cell neoplasms basal cell carcinoma, transitional cell papillomas and carcinomas, adenomas and adenocarcinomas (glands), adenoma, adenocarcinoma, linitis plastica insulinoma, glucagonoma, gastrinoma, vipoma, cholangiocarcinoma, hepatocellular carcinoma, adenoid cystic carcinoma, carcinoid tumor of appendix, prolactinoma, oncocytoma,

hurthle cell adenoma, renal cell carcinoma, grawitz tumor, multiple endocrine adenomas, endometrioid adenoma, adnexal and skin appendage neoplasms, mucoepidermoid neoplasms, cystic, mucinous and serous neoplasms, cystadenoma, pseudomyxoma peritonei, ductal, lobular and medullary neoplasms, acinar cell neoplasms, complex epithelial neoplasms, warthin's tumor, thymoma, specialized gonadal neoplasms, sex cord stromal tumor, thecoma, granulosa cell tumor, arrhenoblastoma, sertoli leydig cell tumor, glomus tumors, paraganglioma, pheochromocytoma, glomus tumor, nevi and melanomas, melanocytic nevus, malignant melanoma, melanoma, nodular melanoma, dysplastic nevus, lentigo maligna melanoma, superficial spreading melanoma, and malignant acral lentiginous melanoma. Sarcoma that can be assessed using the subject methods include without limitation Askin's tumor, botryodies, chondrosarcoma, Ewing's sarcoma, malignant hemangio endothelioma, malignant schwannoma, osteosarcoma, soft tissue sarcomas including: alveolar soft part sarcoma, angiosarcoma, cystosarcoma phyllodes, dermatofibrosarcoma, desmoid tumor, desmoplastic small round cell tumor, epithelioid sarcoma, extraskeletal chondrosarcoma, extraskeletal osteosarcoma, fibrosarcoma, hemangiopericytoma, hemangiosarcoma, kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, rhabdomyosarcoma, and synovialsarcoma. Lymphoma and leukemia that can be assessed using the subject methods include without limitation chronic lymphocytic leukemia/small lymphocytic lymphoma, B-cell prolymphocytic leukemia, lymphoplasmacytic lymphoma (such as waldenström macroglobulinemia), splenic marginal zone lymphoma, plasma cell myeloma, plasmacytoma, monoclonal immunoglobulin deposition diseases, heavy chain diseases, extranodal marginal zone B cell lymphoma, also called malt lymphoma, nodal marginal zone B cell lymphoma (nmzl), follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma, mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, burkitt lymphoma/leukemia, T cell prolymphocytic leukemia, T cell large granular lymphocytic leukemia, aggressive NK cell leukemia, adult T cell leukemia/lymphoma, extranodal NK/T cell lymphoma, nasal type, enteropathy-type T cell lymphoma, hepatosplenic T cell lymphoma, blastic NK cell lymphoma, mycosis fungoides / sezary syndrome, primary cutaneous CD30-positive T cell lymphoproliferative disorders, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis, angioimmunoblastic T cell lymphoma, peripheral T cell lymphoma, unspecified, anaplastic large cell lymphoma, classical Hodgkin lymphomas (nodular sclerosis, mixed cellularity, lymphocyte-rich, lymphocyte depleted or not depleted), and nodular lymphocyte-predominant Hodgkin lymphoma. Germ cell tumors that can be assessed using the subject methods include without limitation germinoma, dysgerminoma, seminoma, nongerminomatous germ cell tumor, embryonal carcinoma, endodermal sinus turmor, choriocarcinoma, teratoma, polyembryoma, and gonadoblastoma. Blastoma includes without limitation nephroblastoma, medulloblastoma, and retinoblastoma. Other cancers include without limitation labial carcinoma, larynx carcinoma, hypopharynx carcinoma, tongue carcinoma, salivary gland carcinoma, gastric carcinoma, adenocarcinoma, thyroid cancer (medullary and papillary thyroid carcinoma), renal carcinoma, kidney parenchyma carcinoma, cervix carcinoma, uterine corpus carcinoma, endometrium carcinoma, chorion carcinoma, testis carcinoma, urinary carcinoma, melanoma, brain tumors such as glioblastoma, astrocytoma, meningioma, medulloblastoma and peripheral neuroectodermal tumors, gall bladder carcinoma, bronchial carcinoma, multiple myeloma, basalioma, teratoma, retinoblastoma, choroidea melanoma, seminoma, rhabdomyosarcoma, craniopharyngeoma, osteosarcoma, chondrosarcoma, myosarcoma, liposarcoma, fibrosarcoma, Ewing sarcoma, and plasmocytoma.

[00263] In a further embodiment, the cancer may be a lung cancer including non-small cell lung cancer and small cell lung cancer (including small cell carcinoma (oat cell cancer), mixed small cell/large cell carcinoma, and combined small cell carcinoma), colon cancer, breast cancer, prostate cancer, liver cancer, pancreas cancer, brain cancer, kidney cancer, ovarian cancer, stomach cancer, skin cancer, bone cancer, gastric cancer, breast cancer, pancreatic cancer, glioma, glioblastoma, hepatocellular carcinoma, papillary renal carcinoma, head and neck squamous cell carcinoma, leukemia, lymphoma, myeloma, or a solid tumor.

[00264] In embodiments, the cancer comprises an acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related cancers; AIDS-related lymphoma; anal cancer; appendix cancer; astrocytomas; atypical teratoid/rhabdoid tumor; basal cell carcinoma; bladder cancer; brain stem glioma; brain tumor (including brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, astrocytomas, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, supratentorial primitive neuroectodermal tumors and pineoblastoma); breast cancer; bronchial tumors; Burkitt lymphoma; cancer of unknown primary site; carcinoid tumor; carcinoma of unknown primary site; central nervous system atypical teratoid/rhabdoid tumor; central nervous system embryonal tumors; cervical cancer; childhood cancers; chordoma; chronic lymphocytic leukemia; chronic myelogenous leukemia; chronic myeloproliferative disorders; colon cancer; colorectal cancer; craniopharyngioma; cutaneous T-cell lymphoma; endocrine pancreas islet cell tumors; endometrial cancer; ependymoblastoma; ependymoma; esophageal cancer; esthesioneuroblastoma; Ewing sarcoma; extracranial germ cell tumor; extragonadal germ cell tumor; extrahepatic bile duct cancer; gallbladder cancer; gastric (stomach) cancer; gastrointestinal carcinoid tumor; gastrointestinal stromal cell tumor; gastrointestinal stromal tumor (GIST); gestational trophoblastic tumor; glioma; hairy cell leukemia; head and neck cancer; heart cancer; Hodgkin lymphoma; hypopharyngeal cancer; intraocular melanoma; islet cell tumors; Kaposi sarcoma; kidney cancer; Langerhans cell histiocytosis; laryngeal cancer; lip cancer; liver cancer; malignant fibrous histiocytoma bone cancer; medulloblastoma; medulloepithelioma; melanoma; Merkel cell carcinoma; Merkel cell skin carcinoma; mesothelioma; metastatic squamous neck cancer with occult primary; mouth cancer; multiple endocrine neoplasia syndromes; multiple myeloma; multiple myeloma/plasma cell neoplasm; mycosis fungoides; myelodysplastic syndromes; myeloproliferative neoplasms; nasal cavity cancer; nasopharyngeal cancer; neuroblastoma; Non-Hodgkin lymphoma; nonmelanoma skin cancer; non-small cell lung cancer; oral cancer; oral cavity cancer; oropharyngeal cancer; osteosarcoma; other brain and spinal cord tumors; ovarian cancer; ovarian epithelial cancer; ovarian germ cell tumor; ovarian low malignant potential tumor; pancreatic cancer; papillomatosis; paranasal sinus cancer; parathyroid cancer; pelvic cancer; penile cancer; pharyngeal cancer; pineal parenchymal tumors of intermediate differentiation; pineoblastoma; pituitary tumor; plasma cell neoplasm/multiple myeloma; pleuropulmonary blastoma; primary central nervous system (CNS) lymphoma; primary hepatocellular liver cancer; prostate cancer; rectal cancer; renal cancer; renal cell (kidney) cancer; renal cell cancer; respiratory tract cancer; retinoblastoma; rhabdomyosarcoma; salivary gland cancer; Sézary syndrome; small cell lung cancer; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; squamous neck cancer; stomach (gastric) cancer; supratentorial primitive neuroectodermal tumors; T-cell lymphoma; testicular cancer; throat cancer; thymic carcinoma; thymoma; thyroid cancer; transitional cell cancer; transitional cell cancer of the renal pelvis and ureter; trophoblastic tumor; ureter cancer; urethral cancer;

uterine cancer; uterine sarcoma; vaginal cancer; vulvar cancer; Waldenström macroglobulinemia; or Wilm's tumor.

[00265] The methods of the invention can be used to determine biomarker patterns or biomarker signature sets in a number of tumor types, diseased tissue types, or diseased cells including accessory, sinuses, middle and inner ear, adrenal glands, appendix, hematopoietic system, bones and joints, spinal cord, breast, cerebellum, cervix uteri, connective and soft tissue, corpus uteri, esophagus, eye, nose, eyeball, fallopian tube, extrahepatic bile ducts, other mouth, intrahepatic bile ducts, kidney, appendix-colon, larynx, lip, liver, lung and bronchus, lymph nodes, cerebral, spinal, nasal cartilage, excl. retina, eye, nos, oropharynx, other endocrine glands, other female genital, ovary, pancreas, penis and scrotum, pituitary gland, pleura, prostate gland, rectum renal pelvis, ureter, peritonem, salivary gland, skin, small intestine, stomach, testis, thymus, thyroid gland, tongue, unknown, urinary bladder, uterus, nos, vagina & labia, and vulva,nos.

[00266] In some embodiments, the molecular profiling methods are used to identify a treatment for a cancer of unknown primary (CUP). Approximately 40,000 CUP cases are reported annually in the US. Most of these are metastatic and/or poorly differentiated tumors. Because molecular profiling can identify a candidate treatment depending only upon the diseased sample, the methods of the invention can be used in the CUP setting. Moreover, molecular profiling can be used to create signatures of known tumors, which can then be used to classify a CUP and identify its origin. In an aspect, the invention provides a method of identifying the origin of a CUP, the method comprising performing molecular profiling on a panel of diseased samples to determine a panel of molecular profiles that correlate with the origin of each diseased sample, performing molecular profiling on a CUP sample, and correlating the molecular profile of the CUP sample with the molecular profiling of the panel of diseased samples, thereby identifying the origin of the CUP sample. The identification of the origin of the CUP sample can be made by matching the molecular profile of the CUP sample with the molecular profiles that correlate most closely from the panel of disease samples. The molecular profiling can use any of the techniques described herein, e.g., IHC, FISH, microarray and sequencing. The diseased samples and CUP samples can be derived from a patient sample, e.g., a biopsy sample, including a fine needle biopsy. In one embodiment, DNA microarray and IHC profiling are performed on the panel of diseased samples, DNA microarray is performed on the CUP samples, and then IHC is performed on the CUP sample for a subset of the most informative genes as indicated by the DNA microarray analysis. This approach can identify the origin of the CUP sample while avoiding the expense of performing unnecessary IHC testing. The IHC can be used to confirm the microarray findings.

[00267] The biomarker patterns or biomarker signature sets of the cancer or tumor can be used to determine a therapeutic agent or therapeutic protocol that is capable of interacting with the biomarker pattern or signature set. For example, with advanced breast cancer, immunohistochemistry analysis can be used to determine one or more gene expressed proteins that are overexpressed. Accordingly, a biomarker pattern or biomarker signature set can be identified for advanced stage breast cancer and a therapeutic agent or therapeutic protocol can be identified which is capable of interacting with the biomarker pattern or signature set.

[00268] The biomarker patterns and/or biomarker signature sets can comprise at least one biomarker. In yet other embodiments, the biomarker patterns or signature sets can comprise at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 biomarkers. In some embodiments, the biomarker signature sets or biomarker patterns can comprise at least 15, 20, 30, 40, 50, or 60 biomarkers. In some embodiments, the biomarker signature sets or biomarker patterns can

comprise at least 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000 or 50,000 biomarkers. Analysis of the one or more biomarkers can be by one or more methods. For example, analysis of 2 biomarkers can be performed using microarrays. Alternatively, one biomarker may be analyzed by IHC and another by microarray. Any such combinations of methods and biomarkers are contemplated herein.

[00269] The one or more biomarkers can be selected from the group consisting of, but not limited to: Her2/Neu, ER, PR, c-kit, EGFR, MLH1, MSH2, CD20, p53, Cyclin D1, bcl2, COX-2, Androgen receptor, CD52, PDGFR, AR, CD25, VEGF, HSP90, PTEN, RRM1, SPARC, Survivin, TOP2A, BCL2, HIF1A, AR, ESR1, PDGFRA, KIT, PDGFRB, CDW52, ZAP70, PGR, SPARC, GART, GSTP1, NFKBIA, MSH2, TXNRD1, HDAC1, PDGFC, PTEN, CD33, TYMS, RXRB, ADA, TNF, ERCC3, RAF1, VEGF, TOP1, TOP2A, BRCA2, TK1, FOLR2, TOP2B, MLH1, IL2RA, DNMT1, HSPCA, ERBR2, ERBB2, SSTR1, VHL, VDR, PTGS2, POLA, CES2, EGFR, OGFR, ASNS, NFKB2, RARA, MS4A1, DCK, DNMT3A, EREG, Epiregulin, FOLR1, GNRH1, GNRHR1, FSHB, FSHR, FSHPRH1, folate receptor, HGF, HIG1, IL13RA1, LTB, ODC1, PPARG, PPARGC1, Lymphotoxin Beta Receptor, Myc, Topoisomerase II, TOPO2B, TXN, VEGFC, ACE2, ADH1C, ADH4, AGT, AREG, CA2, CDK2, caveolin, NFKB1, ASNS, BDCA1, CD52, DHFR, DNMT3B, EPHA2, FLT1, HSP90AA1, KDR, LCK, MGMT, RRM1, RRM2, RRM2B, RXRG, SRC, SSTR2, SSTR3, SSTR4, SSTR5, VEGFA, or YES1.

[00270] For example, a biological sample from an individual can be analyzed to determine a biomarker pattern or biomarker signature set that comprises a biomarker such as HSP90, Survivin, RRM1, SSTRS3, DNMT3B, VEGFA, SSTR4, RRM2, SRC, RRM2B, HSP90AA1, STR2, FLT1, SSTR5, YES1, BRCA1, RRM1, DHFR, KDR, EPHA2, RXRG, or LCK. In other embodiments, the biomarker SPARC, HSP90, TOP2A, PTEN, Survivin, or RRM1 forms part of the biomarker pattern or biomarker signature set. In yet other embodiments, the biomarker MGMT, SSTRS3, DNMT3B, VEGFA, SSTR4, RRM2, SRC, RRM2B, HSP90AA1, STR2, FLT1, SSTR5, YES1, BRCA1, RRM1, DHFR, KDR, EPHA2, RXRG, CD52, or LCK is included in a biomarker pattern or biomarker signature set. In still other embodiments, the biomarker hENT1, cMet, P21, PARP-1, TLE3 or IGF1R is included in a biomarker pattern or biomarker signature set.

[00271] The expression level of HSP90, Survivin, RRM1, SSTRS3, DNMT3B, VEGFA, SSTR4, RRM2, SRC, RRM2B, HSP90AA1, STR2, FLT1, SSTR5, YES1, BRCA1, RRM1, DHFR, KDR, EPHA2, RXRG, or LCK can be determined and used to identify a therapeutic for an individual. The expression level of the biomarker can be used to form a biomarker pattern or biomarker signature set. Determining the expression level can be by analyzing the levels of mRNA or protein, such as by microarray analysis or IHC. In some embodiments, the expression level of a biomarker is performed by IHC, such as for SPARC, TOP2A, or PTEN, and used to identify a therapeutic for an individual. The results of the IHC can be used to form a biomarker pattern or biomarker signature set. In yet other embodiments, a biological sample from an individual or subject is analyzed for the expression level of CD52, such as by determining the mRNA expression level by methods including, but not limited to, microarray analysis. The expression level of CD52 can be used to identify a therapeutic for the individual. The expression level of CD52 can be used to form a biomarker pattern or biomarker signature set. In still other embodiments, the biomarkers hENT1, cMet, P21, PARP-1, TLE3 and/or IGF1R are assessed to identify a therapeutic for the individual.

[00272] As described herein, the molecular profiling of one or more targets can be used to determine or identify a therapeutic for an individual. For example, the expression level of one or more biomarkers can be used to determine or identify a therapeutic for an individual. The one or more biomarkers, such as those disclosed herein, can be used to form a biomarker pattern or biomarker signature set, which is used to identify a therapeutic for an individual. In some embodiments, the therapeutic identified is one that the individual has not previously been treated with. For example, a reference biomarker pattern has been established for a particular therapeutic, such that individuals with the reference biomarker pattern will be responsive to that therapeutic. An individual with a biomarker pattern that differs from the reference, for example the expression of a gene in the biomarker pattern is changed or different from that of the reference, would not be administered that therapeutic. In another example, an individual exhibiting a biomarker pattern that is the same or substantially the same as the reference is advised to be treated with that therapeutic. In some embodiments, the individual has not previously been treated with that therapeutic and thus a new therapeutic has been identified for the individual.

[00273] Methods for providing a theranosis of disease include selecting candidate therapeutics for various cancers by assessing a sample from a subject in need thereof (i.e., suffering from a particular cancer). The sample is assessed by performing an immunohistochemistry (IHC) to determine of the presence or level of: AR, BCRP, c-KIT, ER, ERCC1, HER2, IGF1R, MET (also referred to herein as cMet), MGMT, MRP1, PDGFR, PGP, PR, PTEN, RRM1, SPARC, TOPO1, TOP2A, TS, COX-2, CK5/6, CK14, CK17, Ki67, p53, CAV-1, CYCLIN D1, EGFR, E-cadherin, p95, TLE3 or a combination thereof; performing a microarray analysis on the sample to determine a microarray expression profile on one or more (such as at least five, 10, 15, 20, 25, 30, 40, 50, 60, 70 or all) of: ABCC1, ABCG2, ADA, AR, ASNS, BCL2, BIRC5, BRCA1, BRCA2, CD33, CD52, CDA, CES2, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, ECGF1, EGFR, EPHA2, ERBB2, ERCC1, ERCC3, ESR1, FLT1, FOLR2, FYN, GART, GNRH1, GSTP1, HCK, HDAC1, HIF1A, HSP90AA1, IL2RA, HSP90AA1, KDR, KIT, LCK, LYN, MGMT, MLH1, MS4A1, MSH2, NFKB1, NFKB2, OGFR, PDGFC, PDGFRA, PDGFRB, PGR, POLA1, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SPARC, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGFA, VHL, YES1, and ZAP70; comparing the results obtained from the IHC and microarray analysis against a rules database, wherein the rules database comprises a mapping of candidate treatments whose biological activity is known against a cancer cell that expresses one or more proteins included in the IHC expression profile and/or expresses one or more genes included in the microarray expression profile; and determining a candidate treatment if the comparison indicates that the candidate treatment has biological activity against the cancer.

[00274] Assessment can further comprise determining a fluorescent in-situ hybridization (FISH) profile of EGFR, HER2, cMYC, TOP2A, MET, or a combination thereof, comparing the FISH profile against a rules database comprising a mapping of candidate treatments predetermined as effective against a cancer cell having a mutation profile for EGFR, HER2, cMYC, TOP2A, MET, or a combination thereof, and determining a candidate treatment if the comparison of the FISH profile against the rules database indicates that the candidate treatment has biological activity against the cancer.

[00275] As explained further herein, the FISH analysis can be performed based on the origin of the sample. This can avoid unnecessary laboratory procedures and concomitant expenses by targeting analysis of genes that are known to play a role in a particular disorder, e.g., a particular type of cancer. In an embodiment, EGFR,

HER2, cMYC, and TOP2A are assessed for breast cancer. In another embodiment, EGFR and MET are assessed for lung cancer. Alternately, FISH analysis of all of EGFR, HER2, cMYC, TOP2A, MET can be performed on a sample. The complete panel may be assessed, e.g., when a sample is of unknown or mixed origin, to provide a comprehensive view of an unusual sample, or when economies of scale dictate that it is more efficient to perform FISH on the entire panel than to make individual assessments.

[00276] In an additional embodiment, the sample is assessed by performing nucleic acid sequencing on the sample to determine a presence of a mutation of KRAS, BRAF, PIK3CA (also referred to as PI3K), c-Kit, EGFR, or a combination thereof, comparing the results obtained from the sequencing against a rules database comprising a mapping of candidate treatments predetermined as effective against a cancer cell having a mutation profile for KRAS, BRAF, PIK3CA, c-Kit, EGFR, or a combination thereof; and determining a candidate treatment if the comparison of the sequencing to the mutation profile indicates that the candidate treatment has biological activity against the cancer.

[00277] As explained further herein, the nucleic acid sequencing can be performed based on the origin of the sample. This can avoid unnecessary laboratory procedures and concomitant expenses by targeting analysis of genes that are known to play a role in a particular disorder, e.g., a particular type of cancer. In an embodiment, the sequences of PIK3CA and c-KIT are assessed for breast cancer. In another embodiment, the sequences of KRAS and BRAF are assessed for GI cancers such as colorectal cancer. In still another embodiment, the sequences of KRAS, BRAF and EGFR are assessed for lung cancer. Alternately, sequencing of all of KRAS, BRAF, PIK3CA, c-Kit, EGFR can be performed on a sample. The complete panel may be sequenced, e.g., when a sample is of unknown or mixed origin, to provide a comprehensive view of an unusual sample, or when economies of scale dictate that it is more efficient to sequence the entire panel than to make individual assessments.

[00278] SPARC and HSP90

[00279] Secreted Protein Acidic and Rich in Cysteine (SPARC; also referred to as osteonectin; BM-40) is a multifunctional glycoprotein that binds collagen involved in tissue mineralization and repair and extracellular matrix modeling, and is expressed by many different types of cells. It also functions to organize the basement membrane structure that is required for tumor progression. SPARC consists of three modules which each have independent activity and unique properties. Module I is highly acidic, binds calcium ions, interacts with hydroxyapatite, and is involved in the mineralization of cartilage and bone. Module II is cysteine rich and is homologous to a repeated domain in follistatin which binds to activin and inhibin. Module III is the extracellular calcium binding module and had high-affinity calcium binding sites.

[00280] In vitro experiments have shown that SPARC disrupts cell adhesion, promotes changes in cell shape, inhibits the cell cycle, regulates cell differentiation, inactivates cellular responses to certain growth factors, and regulates extracellular matrix metalloprotease production. The overexpression of SPARC is present in malignant tumors and correlates with disease progression and poor prognosis. SPARC has also been reported to be localized in the cytoplasm of various cell types.

[00281] HSP90 proteins are highly conserved molecular chaperones that have key roles in signal transduction, protein folding, protein degradation, and morphologic evolution. HSP90 proteins normally associate with other cochaperones and play important roles in folding newly synthesized proteins or stabilizing and refolding denatured proteins after stress. There are two major cytosolic HSP90 proteins, HSP90AA1, an inducible form,

and HSP90AB1, a constitutive form. Other HSP90 proteins are found in endoplasmic reticulum and mitochondria. Mitocondrial HSP90 is known as TRAP1. In humans, two distinct HSP90 have been discovered to date: HSP89-alpha and HSP89-beta.

[00282] HSP90AA1 protein has a highly conserved N-terminal domain, a charged domain, a middle domain involved in ATPase activity, a second charged domain, and a C-terminal domain. It also has a 4-helical cytokine motif, a gln-rich region, and a C-terminal MEEVD motif characteristic of cytosolic HSP90 proteins. The 854-amino acid HSP90AA1 isoform has an N-terminal extension compared with the 732-amino acid isoform, but is otherwise identical. An HSP90 protein can be chimeric. For example, Chen et al. discovered a CD47/HSP90 chimeric protein (Genomics 86: 627-637, 2005). HSP90 and CD37 associate with CDK4 not bound to D-type cyclins. Pharmacologic inactivation of CDC37/HSP90 function leads to reduced stability of CDK4.

[00283] HSP90 and CDC37 are two additional components of the I-kappa-B kinase (IKK) complex. This complex also contains two catalytic subunits, IKK-alpha and IKK-beta, and a regulatory subunit, NEMO. HSP90 and HSP70 dock onto a specialized tetratricopeptide (TPR) domain in the import receptor TOMM70 at the outer mitochondrial membrane. This interaction serves to deliver a set of preproteins to the receptor for subsequent membrane translocation dependent on the HSP90 ATPase. Disruption of the chaperone/TOMM70 recognition inhibits the import of these preproteins into mitochondria.

[00284] HSP90 plays a role in the conformational maturation of oncogenic signaling proteins, including HER2/ERBB2, AKT (164730), RAF1, BCR-ABL, and mutated p53. HSP90 inhibitors bind to HSP90, and induce the proteasomal degradation of HSP90 client proteins. Although HSP90 is highly expressed in most cells, HSP90 inhibitors selectively kill cancer cells compared to normal cells, and the HSP90 inhibitor 17-allylaminogeldanamycin (17-AAG) exhibited antitumor activity in preclinical models. HSP90 derived from tumor cells has a 100-fold higher binding affinity for 17-AAG than does HSP90 from normal cells. Tumor HSP90 is present entirely in multichaperone complexes with high ATPase activity, whereas HSP90 from normal tissues is in a latent, uncomplexed state.

[00285] HSP90 is an important extracellular mediator of cancer cell invasion. HSP90A is expressed extracellularly on fibrosarcoma and breast cancer cells. HSP90A interacted with MMP2 outside the cell and promoted MMP2 activation, which is critical for tumor invasiveness.

[00286] Endogenous HSP90 interacts directly with BCL6 in diffuse large B-cell lymphomas (DLBCLs) and stabilized BCL6 mRNA and protein. HSP90 and BCL6 are coexpressed in the nuclei of primary DLBCL cells. HSP90 formed a complex with BCL6 at BCL6 target promoters, and pharmacologic inhibition of HSP90 derepresses BCL6 target genes.

[00287] Interdigitating dendritic cells are potent antigen presenting cells found in T-cell areas of peripheral lymphoid tissue. Interdigitating dendritic cell sarcoma (IDCS) is an extremely rare neoplasm that can mimic other primary and metastatic spindle cell neoplasms of lymph nodes. Characterized by S100, CD68, and CD45RB staining, IDCS often presents with metastasis and portends a poor prognosis. For localized disease treated with surgery alone, approximately 50% remain disease-free with a median follow up of 12 months (range two months to 19 years). In contrast, with advanced IDCS, survival rarely exceeds 12 months, despite various treatment modalities including surgical excision, multi-agent systemic chemotherapy, and/or radiotherapy. The co-expression of SPARC and HSP90, as described herein, can be indicative of the presence of IDCS.

[00288] Due to the correlation between the overexpression of SPARC and HSP90 in malignant tissue, it would be beneficial to have a fast and reliable method for determining whether a sample possesses elevated levels of SPARC and HSP90. It would also be advantageous to be able to use such a method for diagnostic, prognostic and theranostic purposes.

[00289] Provided herein are methods of assessing SPARC and HSP90 levels in a biological tissue using molecular profiling. The molecular profiling can also comprise IHC, FISH, microarray, immunoassay, PCR, sequencing, or other molecular profiling techniques as described herein. In embodiments, the methods comprise performing immunohistochemistry on a sample from a subject, e.g., a tissue sample. For example, the methods can include performing immunohistochemistry with a monoclonal or a polyclonal antibody against SPARC or HSP90 on a tissue sample from a subject, and determining whether the monoclonal or polyclonal SPARC or HSP90 antibody is associated with SPARC or HSP90 polypeptide or a fragment thereof in the sample. The detection of SPARC and/or HSP90 in the sample, e.g., an association between the monoclonal or polyclonal SPARC antibody with SPARC polypeptide or fragment or an association between the monoclonal or polyclonal HSP90 antibody with HSP90 polypeptide or fragment, can be indicative of a disease. The disease can be a cancer, such as a malignant tumor.

[00290] The tumor can be any tumor expressing SPARC and/or HSP90 at a level above the normal or healthy level associated with a particular cell or tissue from which the tumor is originated. The tumor can be of any type or origin, such as the types or origins disclosed herein. In one embodiment, the tumor comprises interdigitating dendritic cell sarcoma. In another embodiment, the tumor comprises human renal cell carcinoma.

[00291] The levels of SPARC and HSP90 detected in a sample can be indicative of a disease in the sample. For example, an amount of staining based on the association of a SPARC antibody and SPARC polypeptide or fragment thereof and the amount of HSP90 antibody with a HSP90 polypeptide or fragment, can be indicative of a disease. A value can be applied for the percent of tissue which is stained, with 0% indicating that no tissue was stained and 100% indicating that all of the tissue was stained. For example, a disease can be indicated when at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99% of tissue sample is stained, a disease is indicated. The level can be determined based on the level of SPARC and HSP90 detected in control, or reference, tissue. The control can be, e.g., a tissue from another individual without the disease or a tissue from the same individual but without the disease. In some embodiments, the expression of SPARC and HSP90 are indicative of a disease when SPARC and HSP90 are expressed or detected in at least 30% of the sample.

[00292] The intensity of staining of the tissue sample can also be determined, for example with the use of a light microscope. For example, a scale of staining intensity levels can be from 0 to 4, wherein the levels are 0, 1, 2, 3, 4 (also identified as 0+, 1+, 2+, 3+, 4+, e.g., as in **FIG. 5**) with 4 being the highest intensity level of staining. In some embodiments, a staining intensity of 2+ or more are indicative of a disease. For example, a staining intensity of 2+ or more can be indicative of a cancer or malignancy.

[00293] The percent of tissue stained and the level of staining intensity can be used in combination for determining whether the level of SPARC and HSP90 detected in a tissue sample is indicative of a disease such as cancer. For example, a 2+ or more level of staining for 30% or more of the sample tissue indicating overexpression of SPARC or HSP90 can be indicative of a malignancy. The overexpression can and also used to select a candidate chemotherapeutic agent for treating the malignancy, such as the example presented in **Table 5** for an interdigitating dendritic cell sarcoma:

Table 5: IHC analysis-interdigitating dendritic cell sarcoma

Positive IHC Staining	Conclusion	Specificity	Intensity	%	Potential Agent to be Tried
SPARC	Positive	Specific	3	40	Paclitaxel-albumin bound
HSP90	Positive	Specific	2	70	CNF2024, SNX5422, IPI-504, or a geldanomycin derivative

[00294] If it is determined that the sample from a subject has an elevated SPARC and HSP90 level, a candidate treatment can be selected that is associated with SPARC and HSP90. The candidate treatment can include one or more therapeutic agent. For example, the subject can be treated with a mitotic inhibitor, such as a taxane, a vinca alkaloid, colchicine and/or some other cytotoxic agent. Taxanes can disrupt microtubule function by stabilizing GDP-bound tubulin in the microtubule. Because microtubules are essential to cell division, taxanes thereby inhibit the process of cell division, resulting in a frozen mitosis. On the other hand, the vinca alkaloids destroy mitotic spindles. The subject maybe treated with taxanes such as paclitaxel, nab-paclitaxel, paclitaxel bound to albumin, or docetaxel, or vinca alkyloids such as vincristine, vinblastine, vindesine or vinorelbine, or any agent selected by the rules in **Tables 2**, **3**, or as indicated in **Table 5**. The agents can also be selected according to the drug associations and rules that are found in U.S. Provisional Patent Application 61/427,788, filed December 28, 2010; U.S. Patent Application 12/658,770, filed February 12, 2010; International PCT Patent Application PCT/US2010/000407, filed February 11, 2010; and International PCT Patent Application PCT/US2010/54366, filed October 27, 2010; all of which applications are incorporated by reference herein in their entirety. See e.g., "Table 4: Rules Summary for Treatment Selection" of PCT/US2010/54366 and "Table 4: Rules Summary for Treatment Selection" of U.S. 61/427,788.

[00295] The agent can also be an inhibitor of HSP90. Inhibition of HSP90 can lead to inactivation and/or degradation of tumor related proteins including without limitation transmembrane tyrosine kinases (Her-2, EGFR), metastable signaling proteins (Akt, Raf-1 and IKK), mutated signaling proteins (p53, v-Src), chimeric signaling proteins (Bcr-Abl), cell cycle regulators (Cdk4, Cdk6), and steroid receptors (androgen, estrogen, and progesterone receptors). See, e.g., Pearl et al., The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J. 2008 Mar 15;410(3):439-53. Geldanamycin is a benzoquinone ansamycin antibiotic that binds to HSP90 to interfere with its action. Geldanamycin derivatives include 17-*N*-Allylamino-17-demethoxygeldanamycin (17-AAG) and 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG). HSP90 inhibitors further include without limitation IPI-504 (retaspimycin), 17-AAG (tanespimycin, KOS-953), BIIB021 (CNF2024), BIIB028, SNX-5422, Ganetespib STA-9090, AUY922, AT13387 and cisplatin. Natural inhibitors of HSP90 include without limitation herbimycin, radicicol, novobiocin, coumermycin A1, clorobiocin, epigallocatechin gallate (EGCG), taxol, pochonin, derrubone, gedunin, and celastrol. See, e.g., Donnelly and Blagg, Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket. Curr Med Chem. 2008;15(26):2702-17; Amolins and Blagg, Natural product inhibitors of Hsp90: potential leads for drug discovery. Mini Rev Med Chem. 2009 Feb;9(2):140-52; Sgobba and Rastelli, Structure-based and in silico design of Hsp90 inhibitors. Chem Med Chem. 2009 Sep;4(9):1399-409; Biamonte et al., Heat shock protein 90: inhibitors in clinical trials. J Med Chem. 2010 Jan 14;53(1):3-17; Taldone and Chiosis, Purine-scaffold Hsp90 inhibitors. Curr Top Med Chem. 2009;9(15):1436-46; Li et al., New developments in Hsp90 inhibitors as anti-cancer therapeutics: mechanisms, clinical perspective and more

potential. Drug Resist Updat. 2009 Feb-Apr;12(1-2):17-27; each of which is incorporated by reference herein in its entirety. Derivatives of any of these inhibitors, e.g., derivatives that increase bioavailability, selectivity or specificity, can also be used with the methods of the invention. Because the methods of the invention identify therapeutic targets, the methods of identifying HSP90 overactivation in a tumor can be used to select any HSP90 inhibitor as a candidate treatment that is available at the time the method is performed for consideration by the treating physician.

[00296] The invention further provides methods for detecting an elevated level of SPARC and HSP90 in biological samples using immunohistochemical staining with monoclonal and/or polyclonal antibodies. A flow chart depicting exemplary embodiments of the method for detecting overexpression of SPARC is shown in **FIG. 2**. The process in **FIG. 2** can similarly be used for IHC to detect HSP90 by using anti-HSP90 antibodies. The method generally includes providing a slide (such as a microscope slide) having a sample contained thereon in step 12 and further processing the sample in step 14 such as deparaffinizing and rehydrating the sample in the event that the sample comprises a formalin fixed paraffin embedded (FFPE) tissue. Other steps for processing the sample may also be performed in step 14 in the event that the sample is a frozen sample or fresh sample. The sample contained on the slide is then rinsed in step 16 with a buffer and/or other aqueous liquids. An exemplary buffer and/or other aqueous liquids used for rinsing the sample may include, but are not limited to, a Tris Buffer.

[00297] The sample contained on the slide is then covered with a peroxide solution or other oxidative agent in step 18 to inhibit or block endogenous peroxidase activity in the cells and/or tissues of the sample. In one embodiment, the sample is covered in 3% H_2O_2 for approximately five minutes. In other embodiments, other endogenous peroxide blocking agents may be used for a longer period of time including time periods for less than five minutes and time periods up to sixty minutes depending upon the blocking agent.

[00298] The sample contained on the slide is then rinsed with buffer and/or other aqueous liquids in step 20 as previously described above with reference to step 16. Following step 20, the sample is covered with a primary SPARC antibody. At this juncture, different SPARC antibodies may be used including monoclonal SPARC antibodies and polyclonal SPARC antibodies. For example, in one exemplary embodiment of the method of the present invention, the sample may be covered with a monoclonal SPARC antibody in step 22 such as, for example, a mouse monoclonal SPARC antibody. However, other SPARC antibodies may be used including, but not limited to those derived from rabbit, goat, human, etc. In the exemplary embodiment using a mouse monoclonal SPARC antibody, the sample is covered with the mouse monoclonal SPARC antibody diluted in a Tris based diluent or a negative control agent for approximately 30 minutes. However, depending upon the type of monoclonal antibody used, the sample may be covered with the monoclonal antibody for a period of time ranging between five and 120 minutes. Next, the sample is rinsed in step 24 with a buffer and/or other aqueous liquids as previously described above in step 16. After rinsing, the sample is covered with a secondary detection reagent in step 28. Depending upon the monoclonal SPARC antibody used in step 22, the sample may be covered with a monoclonal antibody probe, a secondary antibody reagent, or a polymer detection reagent for a period of time between 5 and 120 minutes. In the example described above which used a mouse monoclonal SPARC antibody in step 22, the sample is covered with a monoclonal antibody probe such as biotinylated goat anti-mouse antibody for twenty minutes in step 28.

[00299] In another exemplary embodiment of the method of the present invention, the sample is covered with a polyclonal SPARC antibody in step 30 after being rinsed in step 20. In one exemplary embodiment, the

polyclonal SPARC antibody used is rat anti-SPARC antibody and is diluted in a Tris diluent or a negative control agent, or other suitable carrier solution, which covers the sample for approximately thirty minutes. Again, depending upon the polyclonal SPARC antibody used, a sample may be covered with the polyclonal SPARC antibody for a period of time ranging between five and 120 minutes. Next, the sample is rinsed in step 24 with a buffer or other aqueous liquid as previously described in step 16 and then covered with a secondary detection reagent in step 28. In one exemplary embodiment, the sample may be covered with a polyclonal antibody probe that is a goat anti-rat IgG antibody reagent for approximately 15 minutes. Again, depending upon the type of secondary detection reagent used, the sample may be covered with a secondary detection reagent for a period of time ranging between approximately 5 to 120 minutes.

[00300] Next, the sample is rinsed in step 34 with a buffer or aqueous liquid as previously described in step 16 after being covered with a secondary detection reagent in step 28. A horseradish peroxidase conjugate, and enzymatic agent, or a catalytic agent is then used to cover the sample in step 36. After rinsing the sample in step 38 with a buffer or other aqueous liquid as previously described above with reference to step 16, the sample is covered with a first volume of a 3, 3'-diaminobenzidine (DAB) containing solution and/or a chromogen containing solution in step 40. Next, the sample is covered with a second volume of a DAB containing solution and/or a chromogen containing solution in step 42. Depending upon the solution used, the sample may be covered with a DAB containing solution and/or a chromogen containing solution in steps 40 and 42 for a time period of between approximately one to 60 minutes. In one exemplary embodiment, the sample was covered with two separate volumes of DAB containing solution for approximately ten minutes each with no rinse steps between applying those volumes.

[00301] After applying a DAB containing solution and/or a chromogen containing solutions in steps 40 and 42, the sample is rinsed with a buffer or other aqueous liquid in step 44 as previously described above with reference to step 16. After rinse step 44, the sample containing slide is repeatedly rinsed in water in step 46. Step 46 may be performed by repeatedly dipping the sample containing slide into distilled water until the slide appears clear. In addition, an optional step 48 may be carried out which includes covering the sample with a counterstain, such as a hematoxylin counterstain, and then rinsing the sample prior to step 44 in which the sample is repeatedly rinsed with water.

[00302] The sample contained on the slide is then dehydrated in step 50 and a cover slip is applied over the sample in step 52 before using a microscope to examine the slide in step 54 so that one examining the slide can detect an elevated level of SPARC. The process can be performed similarly to detect HSP90 by substituting HSP90 antibodies for the SPARC antibodies.

[00303] It will be understood by those of ordinary skill in the art that a number of variations may be made to the steps outlined in **FIG. 2** to enable one to detect an overexpression of SPARC and HSP90.

[00304] **FIG. 3** is a series of photographs showing overexpression of SPARC in patient tissue samples after carrying out the monoclonal and polyclonal methods of the present invention for detecting an elevated level of SPARC compared to a positive control for SPARC and a negative control for SPARC. The method of the present invention for detecting an elevated level of SPARC using a polyclonal antibody is generally described with reference to **FIG. 2** but includes the following details regarding some of the method steps: 1) the sample used comprised a formalin fixed paraffin embedded (FFPE) biological tissue; 2) step 14 in **FIG. 2** comprised deparaffinizing and rehydrating the FFPE tissue sample; 3) step 18 comprised covering the tissue sample with

3% peroxide for approximately 5 minutes; 4) applying the primary antibody in step 30 comprised covering the tissue sample with a rat Anti-SPARC polyclonal antibody diluted in a Tris based diluent for approximately 30 minutes; 5) applying the secondary antibody reagent in step 32 comprised covering the sample with a goat Anti-Rat IgG antibody reagent for approximately fifteen minutes; 6) step 36 comprised covering the sample with a streptavidin horseradish peroxidase for approximately fifteen minutes; 7) steps 40 and 42 comprised the steps of applying a same volume of DAB containing solution for approximately ten minutes each; and, 8) the sample was covered with a hematoxylin counterstain in step 48 for approximately two minutes.

[00305] The method of the present invention for detecting overexpression of SPARC using a monoclonal antibody can further include the steps described with reference to **FIG. 2** along with the following optional details relating to some of those steps: 1) the sample used comprised a formalin fixed paraffin embedded (FFPE) biological tissue; 2) step 14 in **FIG. 2** comprised deparaffinizing and rehydrating the FFPE tissue sample; 3) step 18 comprised covering the tissue sample with 3% peroxide for approximately 5 minutes; 4) applying the primary antibody in step 30 comprised covering the tissue sample with a monoclonal SPARC antibody diluted in a Tris based diluent for approximately 30 minutes; 5) covering the tissue sample in step 28 comprises covering the tissue sample with biotinylated goat anti-mouse antibody for approximately 20 minutes; 6) step 36 comprised covering the sample with a horseradish peroxidase for approximately twenty minutes; 7) steps 40 and 42 comprised the steps of applying a same volume of DAB containing solution for approximately ten minutes each; and, 8) the sample was covered with a hematoxylin counterstain in step 48 for approximately two minutes.

[00306] **FIGS. 4A-4B** show a table with results of the exemplary monoclonal and polyclonal embodiments of the present invention for detecting an elevated level of SPARC in a sample set of biological tissues in accordance with the description of detailed steps outlined in the preceding paragraph. Two different values were applied to both the tumor tissue and the stroma (i.e., the connective tissue surrounding the tumor) for each patient using both a monoclonal antibody for detecting an elevated level of SPARC and a polyclonal antibody for detecting an elevated level of SPARC as described herein. An intensity of staining level was applied using a light microscope with the range of staining intensity levels being 0, 1, 2, 3, 4 (also identified as 0+, 1+, 2+, 3+, 4+ in **FIG. 5**) with 4 being the highest intensity level of staining. A value was also applied for the percent of tumor tissue which was stained and the percent of stroma that was stained with 0% indicating that none of the tissue was stained and 100% indicating that all of the tissue was stained. A 2+ or more level of staining for 30% or more of the sample tissue is determined to indicate overexpression of SPARC. These optional steps and detection threshold levels are similarly applied for analysis of HSP90 levels using HSP90 antibodies.

[00307] The asterisks in **FIG. 4** identify those tissue samples where the type of antibody singly contributed to the overexpression of SPARC. For example, in the first case identified as MP-TN06-06227, overexpression of SPARC was detected using a polyclonal anti-SPARC antibody but no overexpression was identified with the monoclonal anti-SPARC antibody. Accordingly, the polyclonal antibody described with reference to **FIG. 3** above was singly responsible for identifying the overexpression of SPARC. In another example, the case identified as MP-TN08-08177 in **FIG. 4**, indicated overexpression of SPARC when using the monoclonal anti-SPARC antibody but no overexpression was identified with the polyclonal anti-SPARC antibody. Accordingly, the monoclonal antibody described with reference to **FIG. 3** above was singly responsible for identifying the overexpression of SPARC.

[00308] **FIG. 5** illustrates an exemplary scoring chart which can be used for assigning an overall score to a sample after carrying out the methods of the invention for detecting an elevated level of SPARC or HSP90. The combination of the staining intensity of the sample along with the percent of cells in the sample that was stained correlate to one of four overall scores, namely negative, weakly positive, moderately positive, or strongly positive.

[00309] A flow chart depicting an exemplary embodiment of a method for selecting a candidate treatment for a malignancy 80 is shown in **FIG. 6**. First, a malignant sample is obtained in step 82. Next, a determination is made as to whether the sample possesses an elevated level of SPARC and/or HSP90 as compared to a reference in step 84. The level of SPARC and/or HSP90 can be determined by carrying out the method steps shown in, and described with reference to, **FIG. 2**. The levels of SPARC and/or HSP90 can also be determined using other molecular profiling methods as described herein, including without limitation ISH, FISH, PCR, RT-PCR, immunoassays, microarray analysis, sequencing, or any combination or variations thereof. If it is determined that the sample has an elevated SPARC and/or HSP90 level, the patient having the malignancy relating to the sample can be treated with a taxane, a vinca alkaloid, and/or some other cytotoxic agent in step 86. Step 86 may include selecting a candidate a patient's malignancy with paclitaxel, nab-paclitaxel, or a paclitaxel bound to albumin. The treatment can include an HSP90 inhibitor. In some embodiments, the process is performed for both SPARC and HSP90.

[00310] **FIG. 7** is a flow chart showing an exemplary embodiment of a method for diagnosing or prognosing a malignancy 90 in accordance with the methods of the present invention. First, a test sample must be obtained from a patient in step 92. Next, the test sample from the patient is used to determine whether the test sample has an elevated SPARC and/or HSP90 level as compared to a reference in step 94. The level of SPARC and/or HSP90 can be determined by carrying out the method steps shown in, and described with reference to, **FIG. 2**. The levels of SPARC and/or HSP90 can also be determined using other molecular profiling methods as described herein, including without limitation ISH, FISH, PCR, RT-PCR, immunoassays, microarray analysis, sequencing, or any combination or variations thereof. Finally, a determination is made that the test sample is malignant if the test sample possesses an elevated level of SPARC and/or HSP90 in step 96. When performing IHC according to the subject methods, an elevated level of SPARC and/or HSP90 can be identified as a 2+ or more staining intensity level of 30% or more of the sample. In some embodiments, the process is performed for both SPARC and HSP90. Depending on the setting, the diagnosis of malignancy can be made if: 1) SPARC is overexpressed, 2) HSP90 is overexpressed; or 3) both SPARC and HSP90 are overexpressed.

[00311] When performing a prognosis, the prognosis can depend on the level of SPARC or HSP90 observed in the sample. For example, a higher level of SPARC and/or HSP90 as compared to a reference may indicate a worse prognosis. Similarly, a lower level of SPARC and/or HSP90 as compared to a reference may indicate a better prognosis.

[00312] The subject methods can be performed to monitor a treatment efficacy. For example, the levels of SPARC or HSP90 can be observed in samples from subject taken over a time course during a treatment for a malignancy. The samples can be collected at appropriate periods to observe a treatment effect, including without limitation before and after surgery, before and after initiation of a therapeutic regimine, or over the course of therapy. Reduced levels of SPARC and/or HSP90 over time can indicate that the subject is responding positively to the therapy. Increasing or steady levels of SPARC and/or HSP90 over time can indicate that the

subject is not responding to the therapy. In such cases, the molecular profiling methods of the invention can be used to select another candidate treatment for the malignancy.

[00313] Various methods can also be used to detect SPARC and HSP90. For example, FISH, PCR, (qPCR, RT-PCR, etc) or sequencing can be used to detect SPARC or HSP90 nucleic acids. Examples of sequencing methods include, but are not limited to, sequencing by ligation of dye-modified probes (including cyclic ligation and cleavage), pyrosequencing, single-molecule sequencing, Sanger sequencing, next gen sequencing and gene expression analysis by massively parallel signature sequencing. SPARC and HSP90 proteins can be detected by immunoassays, Western blotting, gel electrophoresis, 2-D electrophoresis, flow cytometry, microbeads, mass spectrometry, and the like. These and other appropriate methods are described further herein.

EXAMPLES

Example 1: IHC and Microarray Testing of over 500 Patients

[00314] Immunohistochemistry (IHC) and expression microarray analysis was performed on diseased tissue from 544 patients whose diseased tissue samples underwent IHC testing and 540 patients whose diseased tissue samples underwent gene microarray testing in accordance with IHC and expression microarray testing as described herein. The patients were all in advanced stages of disease. Biomarker patterns or biomarker signature sets were obtained for a number of tumor types, diseased tissue types, or diseased cells including adipose, adrenal cortex, adrenal gland, adrenal gland – medulla, appendix, bladder, blood vessel, bone, bone cartilage, brain, breast, cartilage, cervix, colon, colon sigmoid, dendritic cells, skeletal muscle, endometrium, esophagus, fallopian tube, fibroblast, gallbladder, kidney, larynx, liver, lung, lymph node, melanocytes, mesothelial lining, myoepithelial cells, osteoblasts, ovary, pancreas, parotid, prostate, salivary gland, sinus tissue, skeletal muscle, skin, small intestine, smooth muscle, stomach, synovium, joint lining tissue, tendon, testis, thymus, thyroid, uterus, and uterus corpus.

[00315] Results of the analysis are summarized in **Table 6**. The table identifies biomarkers tagged as targets in order of frequency observed in the tumors using either IHC to detect proteins or microarray to detect mRNA transcript expression.

[00316] In 99 individuals with advanced breast cancer, immunohistochemistry analysis of 20 gene expressed proteins showed that the gene expressed proteins analyzed were overexpressed a total of 367 times and that 16.35% of that total overexpression was attributable to HSP90 overexpression followed by 12.53% of the overexpression being attributable to TOP2A overexpression and 11.17% of the overexpression being attributable to SPARC. In addition, 9.81% of the overexpression was attributable to androgen receptor overexpression, 9.54% of the overexpression was attributable to PDGFR overexpression, and 9.26% of the overexpression was attributable to c-kit overexpression. Accordingly, a biomarker pattern or biomarker signature set can be identified for advanced stage breast cancer and a therapeutic agent or therapeutic protocol can be identified which is capable of interacting with the biomarker pattern or signature set.

[00317] Another biomarker pattern or biomarker signature set for advanced stage breast cancer was found from from the microarray data analysis. For example, in 100 individuals with advanced breast cancer, gene microarray analysis of 64 genes showed that the genes analyzed exhibited a change in expression a total of 1,158 times and that 6.39% of that total change in expression was attributable to SSTR3 change in expression followed by 5.79% of the change in expression being attributable to VDR change in expression and 5.35% of the change in expression being attributable to BRCA2 change in expression. Accordingly, another biomarker

pattern or biomarker signature set can be identified for advanced stage breast cancer and another therapeutic agent or therapeutic protocol can be identified which is capable of interacting with this biomarker pattern or signature set.

Table 6: Overexpression of Genes and Gene Products

Gene	IHC Analysis		Microarray Analysis	
	Number of samples where biomarker overexpressed	% of overexpressed observations	Number of samples where biomarker overexpressed	% of overexpressed observations
ADA			247	4.0%
AR	78	4.5%	38	0.6%
ASNS			71	1.2%
ASNS			0	0.0%
BRCA1			61	1.0%
BRCA2			114	1.9%
CD25	11	0.6%		
CD52	9	0.5%	4	0.1%
CDW52			0	0.00%
CES2			59	1.0%
COX-2	5	0.3%		
Cyclin D1	0	0.0%		
DCK			148	2.4%
DHFR			54	0.9%
DNMT1			45	0.7%
DNMT3A			196	3.2%
DNMT3B			256	4.2%
EGFR	284	16.6%	76	1.2%
EPHA2			35	0.6%
ERBB2			28	0.5%
ER	60	3.5%		
ESR1			46	0.7%
FLT1			92	1.5%
GART			253	4.1%
GNRH1			1	0.0%
HER2/Neu	64	3.7%		
HIFIA			234	3.8%
HSP90AA1	285	16.6%	111	1.8%
HSPCA			15	0.2%
IL2RA			0	0.0%
KDR			34	0.6%
KIT	148	8.6%	52	0.8%
LCK			0	0.0%
MGMT			303	4.9%
MLH1	11	0.6%	14	0.2%
MSH2	37	2.2%	42	0.7%
NFKB1			205	3.3%
NFKB2			160	2.6%
NFKBIA			261	4.2%
PDGFC			6	0.1%
PDGFRA			39	0.6%
PDGFRB			159	2.6%
PDGFR	179	10.4%		
PGR			17	0.3%
PR	61	3.6%		
PRMI	1	0.1%		
PTEN	14	0.8%	16	0.3%

PTGS2			90	1.5%
RARA			2	0.0%
RRM1			39	0.6%
RRM2			126	2.0%
RRM2B			93	1.5%
RXRG			25	0.4%
SPARC	244	14.2%	143	2.3%
SRC			96	1.6%
SSTR1			145	2.4%
SSTR2			87	1.4%
SSTR3			314	5.1%
SSTR4			136	2.2%
SSTR5			92	1.5%
Survivin	0	0.0%		
TOP1			242	3.9%
TOP2A	224	13.1%	236	3.8%
TOP2B			222	3.6%
TYMS			68	1.1%
VDR			214	3.5%
VEGF			17	0.3%
VEGFA			162	2.6%
VHL			30	0.5%
YES1			77	1.3%
ZAP70			0	0.0%

Example 2: IHC Testing of over 1300 Patients

[00318] Immunohistochemistry was performed on diseased tissue from 1392 patients in accordance with IHC testing as described herein. The patients were all in advanced stages of disease. Biomarker patterns or biomarker signature sets were obtained in a number of tumor types, diseased tissue types, or diseased cells including accessory, sinuses, middle and inner ear, adrenal glands, appendix, hematopoietic system, bones and joints, spinal cord, breast, cerebellum, cervix uteri, connective and soft tissue, corpus uteri, esophagus, eye, nose, eyeball, fallopian tube, extrahepatic bile ducts, other mouth, intrahepatic bile ducts, kidney, appendix-colon, larynx, lip, liver, lung and bronchus, lymph nodes, cerebral, spinal, nasal cartilage, excl. retina, eye, nos, oropharynx, other endocrine glands, other female genital, ovary, pancreas, penis and scrotum, pituitary gland, pleura, prostate gland, rectum renal pelvis, ureter, peritonem, salivary gland, skin, small intestine, stomach, testis, thymus, thyroid gland, tongue, unknown, urinary bladder, uterus, nos, vagina & labia, and vulva,nos.

[00319] In 254 individuals with advanced breast cancer, immunohistochemistry analysis of 19 gene expressed proteins showed that the gene expressed proteins analyzed were overexpressed a total of 767 times and that 13.43% of that total overexpression was attributable to SPARC overexpression followed by 12.26% of the overexpression being attributable to c-kit overexpression and 11.47% of the overexpression being attributable to EGFR. In addition, 11.34% of the overexpression was attributable to androgen receptor overexpression, 11.08% of the overexpression was attributable to HSP90 overexpression, and 10.43% of the overexpression was attributable to PDGFR overexpression. Accordingly, a biomarker pattern or biomarker signature set can be identified for advanced stage breast cancer and a therapeutic agent or therapeutic protocol can be identified which is capable of interacting with the biomarker pattern or signature set.

[00320] **Table** 7 shows protein biomarkers tagged as targets in order of frequency in all tissues tested. Immunohistochemistry of the 19 gene expressed proteins showed that the 19 gene expressed proteins were

tagged 3878 times as targets in the various tissues tested and that EGFR was the gene expressed protein that was overexpressed the most frequently followed by SPARC.

Table 7: Overexpression of Gene Products using IHC

Gene	Number of Times Flagged as Target	% of Flagged Targets
EGFR	731	18.85%
SPARC	569	14.67%
HSP90	483	12.45%
PDGFR	439	11.32%
c-kit	411	10.60%
TOP2A	348	8.97%
Androgen Receptor	187	4.82%
PR	153	3.95%
Her2/Neu	143	3.69%
ER	139	3.58%
COX-2	91	2.35%
CD25	45	1.16%
MSH2	41	1.06%
CD52	40	1.03%
PTEN	33	0.85%
MLH1	13	0.34%
Survivin	7	0.18%
RRM1	4	0.10%
Cyclin D1	1	0.03%

Example 3: Microarray Testing of over 300 Patients

[00321] Expression microarray analysis was performed on diseased tissue from 379 patients whose diseased tissue underwent gene microarray testing in accordance microarray testing as described herein. The patients were all in advanced stages of disease. The data show biomarker patterns or biomarker signature sets in a number of tumor types, diseased tissue types, or diseased cells including accessory, sinuses, middle and inner ear, adrenal glands, anal canal and anus, appendix, blood, bone marrow & hematopoietic sys, bones and joints, brain & cranial nerves and spinal cord (excl. ventricle & cerebellum), breast, cerebellum, cervix uteri, connective & soft tissue, corpus uteri, esophagus, eye,nos, eyeball, fallopian tube, gallbladder & extrahepatic bile ducts, gum,floor of mouth & other mouth, intrahepatic bile ducts, kidney, large intestine (excl. appendix-colon), larynx, lip, liver, lung & bronchus, lymph nodes, meninges (cerebral,spinal), nasal cavity (including nasal cartilage), orbit & lacrimal gland (excl. retina, eye,nos), oropharynx, other endocrine glands, other fenale genital, ovary, pancreas, penis & scrotum, pituitary gland, pleura, prostate gland, rectum, renal pelvis & ureter, retroperitoneum & peritoneum, salivary gland, skin, small intestine, stomach, testis, thymus, thyroid gland, tongue, unknown, unspecified digestive organs, urinary bladder, uterus,nos, vagina & labia, and vulva,nos.

[00322] For example, in 168 individuals with advanced breast cancer, microarray analysis of 63 genes showed that the genes analyzed were either overexpressed or underexpressed a total of 1863 times and that 5.05% of that total change in expression was attributable to SSTR3 change in expression followed by 4.83% of the change in expression being attributable to NKFBIA change in expression and 4.62% of the change in expression being attributable to VDR. In addition, 4.35% of the change in expression was attributable to MGMT change in expression, 4.19% of the change in expression was attributable to ADA change in expression, and 3.97% of the change in expression was attributable to CES2 change in expression.

[00323] **Table 8** shows biomarkers as targets in order of frequency in all tissues that were tested.

Table 8: Overexpression of Gene Products using Microarray Analysis

Grand Total	% in tumor type	Gene
9065	100.00%	Grand Total
398	4.39%	MGMT
396	4.37%	SSTR3
396	4.37%	TOP1
395	4.36%	NFKBIA
384	4.24%	TOP2A
379	4.18%	ADA
369	4.07%	TOP2B
366	4.04%	GART
346	3.82%	HIF1A
339	3.74%	DNMT3B
321	3.54%	NFKB2
318	3.51%	VDR
278	3.07%	NFKB1
267	2.95%	DNMT3A
260	2.87%	SPARC
229	2.53%	PDGFRB
213	2.35%	CES2
182	2.01%	DCK
181	2.00%	VEGFA
167	1.84%	SSTR4
159	1.75%	RRM2
152	1.68%	SSTR1
151	1.67%	DNMT1
138	1.52%	VEGF
136	1.50%	SRC
126	1.39%	EGFR
120	1.32%	BRCA2
114	1.26%	RRM2B
111	1.22%	HSP90AA1
102	1.13%	ASNS
101	1.11%	SSTR2
100	1.10%	FLT1
100	1.10%	PTGS2
96	1.06%	SSTR5
96	1.06%	TYMS
88	0.97%	KIT
87	0.96%	ESR1
81	0.89%	HSPCA
79	0.87%	YES1
76	0.84%	BRCA1
71	0.78%	PDGFRA
66	0.73%	ERBB2
65	0.72%	AR
61	0.67%	RRM1
58	0.64%	DHFR
56	0.62%	VHL
49	0.54%	MSH2
41	0.45%	PDGFC
39	0.43%	KDR
37	0.41%	EPHA2
26	0.29%	RXRG
22	0.24%	PGR
21	0.23%	PTEN
16	0.18%	CDW52
14	0.15%	MLH1
9	0.10%	RARA
6	0.07%	IL2RA
4	0.04%	CD52
2	0.02%	LCK
2	0.02%	ZAP70
1	0.01%	ASNS
1	0.01%	ERCC3
1	0.01%	GNRH1

Example 4: Molecular profiling to find targets and select treatments for refractory cancers

[00324] This Example demonstrates the use of molecular profiling according to the invention to identify candidate treatments for patients with refractory cancers. The primary objective was to compare progression free survival (PFS) using a treatment regimen selected by molecular profiling with the PFS for the most recent regimen the patient progressed on (e.g. patients are their own control) (**FIG. 8**). The molecular profiling approach was deemed of clinical benefit for the individual patient who had a PFS ratio (PFS on molecular profiling selected therapy/PFS on prior therapy) of ≥1.3.

[00325] The study was also performed to determine the frequency with which molecular profiling by IHC, FISH and microarray yielded a target against which there is a commercially available therapeutic agent and to determine response rate (RECIST) and percent of patients without progression or death at 4 months.

[00326] The study was conducted in 9 centers throughout the United States. An overview of the method is depicted in **FIG. 9**. As can be seen in **FIG. 9**, the patient was screened and consented for the study. Patient eligibility was verified by one of two physician monitors. The same physicians confirmed whether the patients had progressed on their prior therapy and how long that PFS (TTP) was. A tumor biopsy was then performed, as discussed below. The tumor was assayed using IHC, FISH (on paraffin-embedded material) and microarray (on fresh frozen tissue) analyses.

[00327] The results of the IHC/FISH and microarray were given to two study physicians who in general used the following algorithm in suggesting therapy to the physician caring for the patient: 1) IHC/FISH and microarray indicated same target was first priority; 2) IHC positive result alone next priority; and 3) microarray positive result alone the last priority.

[00328] The patient's physician was informed of the suggested treatment and the patient was treated with the suggested agent(s) (package insert recommendations). The patient's disease status was assessed every 8 weeks and adverse effects were assessed by the NCI CTCAE version 3.0.

[00329] To be eligible for the study, the patient was required to: 1) provide informed consent and HIPAA authorization; 2) have any histologic type of metastatic cancer; 3) have progressed by RECIST criteria on at least 2 prior regimens for advanced disease; 4) be able to undergo a biopsy or surgical procedure to obtain tumor samples; 5) be ≥18 years, have a life expectancy > 3 months, and an Eastern Cooperative Oncology Group (ECOG) Performance Status or 0-1; 6) have measurable or evaluable disease; 7) be refractory to last line of therapy (documented disease progression under last treatment; received ≥6 weeks of last treatment; discontinued last treatment for progression); 8) have adequate organ and bone marrow function; 9) have adequate methods of birth control; and 10) if CNS metastases then adequately controlled. The ECOG performance scale is described in Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982, which is incorporated by reference in its entirety. Before molecular profiling was performed, the principal investigator at the site caring for the patient must designate what they would treat the patient with if no molecular profiling results were available.

[00330] ***Methods***

[00331] All biopsies were performed at local investigators' sites. For needle biopsies, 2-3 18 gauge needle core biopsies were performed. For DNA microarray (MA) analysis, tissue was immediately frozen and shipped on dry ice via FedEx to a central CLIA certified laboratory, Caris MPI in Phoenix, Arizona. For IHC, paraffin

blocks were shipped on cold packs. IHC was considered positive for target if 2+ in ≥ 30% of cells. The MA was considered positive for a target if the difference in expression for a gene between tumor and control organ tissue was at a significance level of $p \leq 0.001$.

[00332] Ascertainment of the Time to Progression to Document the Progression-Free Survival Ratio

[00333] Time to progression under the last line of treatment was documented by imaging in 58 patients (88%). Among these 58 patients, documentation by imaging alone occurred in 49 patients (74%), and documentation by imaging with tumor markers occurred in nine patients (14%; ovarian cancer, n 3; colorectal, n 1; pancreas, n 1; prostate, n 3; breast, n 1). Patients with clinical proof of progression were accepted when the investigator reported the assessment of palpable and measurable lesions (i.e., inflammatory breast cancer, skin/subcutaneous nodules, or lymph nodes), which occurred in six patients (9%). One patient (2%) with prostate cancer was included with progression by tumor marker. In one patient (2%) with breast cancer, the progression was documented by increase of tumor marker and worsening of bone pain. The time to progression achieved with a treatment based on molecular profiling was documented by imaging in 44 patients (67%) and by clinical events detected between two scheduled tumor assessments in 20 patients. These clinical events were reported as serious adverse events related to disease progression (e.g., death, bleeding, bowel obstruction, hospitalization), and the dates of reporting were censored as progression of disease. The remaining two patients were censored at the date of last follow-up.

[00334] IHC/FISH

[00335] For IHC studies, the formalin fixed, paraffin embedded tumor samples had slices from these blocks submitted for IHC testing for the following proteins: EGFR, SPARC, C-kit, ER, PR, Androgen receptor, PGP, RRM1, TOPO1, BRCP1, MRP1, MGMT, PDGFR, DCK, ERCC1, Thymidylate synthase, Her2/neu and TOPO2A. IHCs for all proteins were not carried out on all patients' tumors.

[00336] Formalin-fixed paraffin-embedded patient tissue blocks were sectioned (4µm thick) and mounted onto glass slides. After deparaffination and rehydration through a series of graded alcohols, pretreatment was performed as required to expose the targeted antigen.

[00337] Human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) were stained as specified by the vendor (DAKO, Denmark). All other antibodies were purchased from commercial sources and visualized with a DAB biotin-free polymer detection kit. Appropriate positive control tissue was used for each antibody. Negative control slides were stained by replacing the primary antibody with an appropriately matched isotype negative control reagent. All slides were counterstained with hematoxylin as the final step and cover slipped. Tissue microarray sections were analyzed by FISH for EGFR and HER-2/neu copy number per the manufacturer's instructions. FISH for HER-2/neu (was done with the PathVysion HER2 DNA Probe Kit (Abbott Molecular, Abbott Park, IL). FISH for EGFR was done with the LSI EGFR/CEP 7 Probe (Abbott Molecular).

[00338] All slides were evaluated semi-quantitatively by a first pathologist, who confirmed the original diagnosis as well as read each of the immunohistochemical stains using a light microscope. Some lineage immunohistochemical stains were performed to confirm the original diagnosis, as necessary. Staining intensity and extent of staining were determined; both positive, tumor-specific staining of tumor cells and highly positive (≥2+), pervasive (≥30%) tumor specific staining results were recorded. IHC was considered positive for target if staining was ≥ 2+ in ≥ 30% of cells. Rather than look for a positive signal without qualification, this approach

raises the stringency of the cut point such that it would be a significant or more demonstrative positive. A higher positive is more likely to be associated with a therapy that would affect the time to progression. The cut point used (i.e., staining was ≥ 2+ in ≥ 30% of cells) is similar to some cut points used in breast cancer for HER2/neu. When IHC cut points were compared with evidence from the tissue of origin of the cancer, the cut points were equal to or higher (more stringent) than the evidence cut points. A standard 10% quality control was performed by a second pathologist.

[00339] Microarray

[00340] Tumor samples obtained for microarray were snap frozen within 30 minutes of resection and transmitted to Caris-MPI on dry ice. The frozen tumor fragments were placed on a 0.5mL aliquot of frozen 0.5M guanidine isothiocyanate solution in a glass tube, and simultaneously thawed and homogenized with a Covaris S2 focused acoustic wave homogenizer (Covaris, Woburn, MA). A 0.5mL aliquot of TriZol was added, mixed and the solution was heated to 65°C for 5 minutes then cooled on ice and phase separated by the addition of chloroform followed by centrifugation. An equal volume of 70% ethanol was added to the aqueous phase and the mixture was chromatographed on a Qiagen RNeasy column (Qiagen, Germantown, MD). RNA was specifically bound and then eluted. The RNA was tested for integrity by assessing the ratio of 28S to 18S ribosomal RNA on an Agilent BioAnalyzer (Agilent, Santa Clara, CA). Two to five micrograms of tumor RNA and two to five micrograms of RNA from a sample of a normal tissue representative of the tumor's tissue of origin were separately converted to cDNA and then labeled during T7 polymerase amplification with contrasting fluor tagged (Cy3, Cy5) cytidine triphosphate. The labeled tumor and its tissue of origin reference were hybridized to an Agilent H1Av2 60-mer olio array chip with 17,085 unique probes.

[00341] The arrays contain probes for 50 genes for which there is a possible therapeutic agent that would potentially interact with that gene (with either high expression or low expression). Those 50 genes included: ADA, AR, ASNA, BCL2, BRCA2, CD33, CDW52, CES2, DNMT1, EGFR, ERBB2, ERCC3, ESR1, FOLR2, GART, GSTP1, HDAC1, HIF1A, HSPCA, IL2RA, KIT, MLH1, MS4A1, MASH2, NFKB2, NFKBIA, OGFR, PDGFC, PDGFRA, PDGFRB, PGR, POLA, PTEN, PTGS2, RAF1, RARA, RXRB, SPARC, SSTR1, TK1, TNF, TOP1, TOP2A, TOP2B, TXNRD1, TYMS, VDR, VEGF, VHL, and ZAP70.

[00342] The chips were hybridized from 16 to 18 hours at 60°C and then washed to remove non-stringently hybridized probe and scanned on an Agilent Microarray Scanner. Fluorescent intensity data were extracted, normalized, and analyzed using Agilent Feature Extraction Software. Gene expression was judged to be different from its reference based on an estimate of the significance of the extent of change, which was estimated using an error model that takes into account the levels of signal to noise for each channel, and uses a large number of positive and negative controls replicated on the chip to condition the estimate. Expression changes at the level of $p \leq 0.001$ were considered as significantly different.

[00343] Statistical Considerations

[00344] The protocol called for a planned 92 patients to be enrolled of which an estimated 64 patients would be treated with therapy assigned by molecular profiling. The other 28 patients were projected to not have molecular profiling results available because of (a) inability to biopsy the patient; (b) no target identified by the molecular profiling; or (c) deteriorating performance status. Sixty four patients were required to receive molecular profiling treatment in order to reject the null hypothesis (Ho) that: ≤15% of patients would have a PFS ratio of ≥1.3 (e.g. a non-promising outcome).

[00345] Treatment Selection

[00346] Treatment for the patients based on molecular profiling results was selected using the following algorithm: 1) IHC/FISH and microarray indicates same target; 2) IHC positive result alone; 3) microarray positive result alone. The patient's physician was informed of suggested treatment and the patient was treated based on package insert recommendations. Disease status was assessed every 8 weeks. Adverse effects were assessed by NCI CTCAE version 3.0.

[00347] The targets and associated drugs are listed in **Table 9**.

Table 9: Pairings of Targets and Drugs

Potential Target	Agents Suggested as Interacting With the Target
IHC	
EGFR	Cetuximab, erlotinib, gefitinib
SPARC	Nanoparticle albumin-bound paclitaxel
c-KIT	Imatinib, sunitinib, sorafenib
ER	Tamoxifen, aromatase inhibitors, toremifene, progestational agent
PR	Progestational agents, tamoxifen, aromatase inhibitor, goserelin
Androgen receptor	Flutamide, abarelix, bicalutamide, leuprolide, goserelin
PGP	Avoid natural products, doxorubicin, etoposide, docetaxel, vinorelbine
HER2/NEU	Trastuzumab
PDGFR	Sunitinib, imatinib, sorafenib
CD52	Alemtuzumab
CD25	Denileukin diftitox
HSP90	Geldanamycin, CNF2024
TOP2A	Doxorubicin, epirubicin, etoposide
Microarray	
ADA	Pentostatin, cytarabine
AR	Flutamide, abarelix, bicalutamide, leuprolide, goserelin
ASNA	Asparaginase
BCL2	Oblimersen sodium†
BRCA2	Mitomycin
CD33	Gemtuzumab ozogamicin
CDW52	Alemtuzumab
CES-2	Irinotecan
DCK	Gemcitabine
DNMT1	Azacitidine, decitabine
EGFR	Cetuximab, erlotinib, gefitinib
ERBB2	Trastuzumab
ERCC1	Cisplatin, carboplatin, oxaliplatin
ESR1	Tamoxifen, aromatase inhibitors, toremifene, progestational agent
FOLR2	Methotrexate, pemetrexed
GART	Pemetrexed
GSTP1	Platinum
HDAC1	Vorinostat
HIF1α	Bevacizumab, sunitinib, sorafenib
HSPCA	Geldanamycin, CNF2024
IL2RA	Aldesleukin
KIT	Imatinib, sunitinib, sorafenib
MLH-1	Gemcitabine, oxaliplatin
MSH1	Gemcitabine
MSH2	Gemcitabine, oxaliplatin
NFKB2	Bortezomib
NFKB1	Bortezomib
OGFR	Opioid growth factor
PDGFC	Sunitinib, imatinib, sorafenib
PDGFRA	Sunitinib, imatinib, sorafenib

PDGFRB	Sunitinib, imatinib, sorafenib
PGR	Progestational agents, tamoxifen, aromatase inhibitors, goserelin
POLA	Cytarabine
PTEN	Rapamycin (if low)
PTGS2	Celecoxib
RAF1	Sorafenib
RARA	Bexarotene, all-trans-retinoic acid
RXRB	Bexarotene
SPARC	Nanoparticle albumin-bound paclitaxel
SSTR1	Octreotide
TK1	Capecitabine
TNF	Infliximab
TOP1	Irinotecan, topotecan
TOP2A	Doxorubicin, etoposide, mitoxantrone
TOP2B	Doxorubicin, etoposide, mitoxantrone
TXNRD1	Px12
TYMS	Fluorouracil, capecitabine
VDR	Calcitriol
VEGF	Bevacizumab, sunitinib, sorafenib
VHL	Bevacizumab, sunitinib, sorafenib
ZAP70	Geldanamycin, CNF2024

[00348] ***Results***

[00349] The distribution of the patients is diagrammed in **FIG. 10** and the characteristics of the patients shown in **Tables 10** and **11**. As can be seen in **FIG. 10**, 106 patients were consented and evaluated. There were 20 patients who did not proceed with molecular profiling for the reasons outlined in **FIG. 10** (mainly worsening condition or withdrawing their consent or they did not want any additional therapy). There were 18 patients who were not treated following molecular profiling (mainly due to worsening condition or withdrawing consent because they did not want additional therapy). There were 68 patients treated, with 66 of them treated according to molecular profiling results and 2 not treated according to molecular profiling results. One of the two was treated with another agent because the clinician caring for the patient felt a sense of urgency to treat and the other was treated with another aagent because the insurance company would not cover the molecular profiling suggested treatment.

[00350] The median time for molecular profiling results being made accessible to a clinician was 16 days from biopsy (range 8 to 30 days) and a median of 8 days (range 0 to 23 days) from receipt of the tissue sample for analysis. Some modest delays were caused by the local teams not sending the patients' blocks immediately (due to their need for a pathology workup of the specimen). Patient tumors were sent from 9 sites throughout the United States including: Greenville, SC; Tyler, TX; Beverly Hills, CA; Huntsville, AL; Indianapolis, IN; San Antonio, TX; Scottsdale, AZ and Los Angeles, CA.

[00351] **Table 10** details the characteristics of the 66 patients who had molecular profiling performed on their tumors and who had treatment according to the molecular profiling results. As seen in **Table 11**, of the 66 patients the majority were female, with a median age of 60 (range 27-75). The number of prior treatment regimens was 2-4 in 53% of patients and 5-13 in 38% of patients. There were 6 patients (9%), who had only 1 prior therapy because no approved active 2nd line therapy was available. Twenty patients had progressed on prior phase I therapies. The majority of patients had an ECOG performance status of 1.

Table 10: Patient Characteristics (n=66)

Characteristic	**n**	**%**
Gender		
Female	43	65
Male	23	35
Age		
Median (range)	60	(27-75)
Number of Prior Treatments		
2-4*	35	53
5-13	25	38
ECOG		
0	18	27
1	48	73

*Note: 6 patients (9%) had 1 prior

[00352] As seen in **Table 11**, tumor types in the 66 patients included breast cancer 18 (27%), colorectal 11 (17%), ovarian 5 (8%), and 32 patients (48%) were in the miscellaneous categories. Many patients had the more rare types of cancers.

Table 11: Patient Tumor Types (n=66)

Tumor Type	**n**	**%**
Breast	***18***	***27***
Colorectal	***11***	***17***
Ovarian	***5***	***8***
Miscellaneous	***32***	***48***
Prostate	4	6
Lung	3	5
Melanoma	2	3
Small cell (esopha/retroperit)	2	3
Cholangiocarcinoma	2	3
Mesothelioma	2	3
H&N (SCC)	2	3
Pancreas	2	3
Pancreas neuroendocrine	1	1.5
Unknown (SCC)	1	1.5
Gastric	1	1.5
Peritoneal pseudomyxoma	1	1.5
Anal Canal (SCC)	1	1.5
Vagina (SCC)	1	1.5
Cervis	1	1.5
Renal	1	1.5
Eccrine seat adenocarinoma	1	1.5
Salivary gland adenocarinoma	1	1.5
Soft tissue sarcoma (uterine)	1	1.5
GIST (Gastric)	1	1.5
Thyroid-Anaplastic	1	1.5

[00353] *Primary Endpoint: PFS Ratio ≥ 1.3*

[00354] As far as the primary endpoint for the study is concerned (PFS ratio of ≥1.3), in the 66 patients treated according to molecular profiling results, the number of patients with PFS ratio greater or equal to 1.3 was 18 out of the 66 or 27%, 95% CI 17-38% one-sided, one-sample non parametric test p=0.007. The null hypothesis was that ≤15% of this patient population would have a PFS ratio of ≥1.3. Therefore, the null hypothesis is rejected and our conclusion is that this molecular profiling approach is beneficial. **FIG. 11** details the comparison of PFS

on molecular profiling therapy (the bar) versus PFS (TTP) on the patient's last prior therapy (the boxes) for the 18 patients. The median PFS ratio is 2.9 (range 1.3-8.15).

[00355] If the primary endpoint is examined, as shown in **Table 12**, a PFS ratio ≥1.3 was achieved in 8/18 (44%) of patients with breast cancer, 4/11 (36%) patients with colorectal cancer, 1/5 (20%) of patients with ovarian cancer and 5/32 (16%) patients in the miscellaneous tumor types (note that miscellaneous tumor types with PFS ratio ≥1.3 included: lung 1/3, cholangiocarcinoma 1/3, mesothelioma 1/2, eccrine sweat gland tumor 1/1, and GIST (gastric) 1/1).

Table 12: Primary Endpoint – PFS Ratio ≥ 1.3 By Tumor Type

Tumor Type	**Total Treated**	**Number with PFS Ratio ≥ 1.3**	**%**
Breast	18	8	44
Colorectal	11	4	36
Ovarian	5	1	20
Miscellaneous*	32	5	16
Total	**66**	**18**	**27**

*lung 1/3, cholangiocarcinoma ½, mesothelioma ½, eccrine sweat 1/1, GIST (gastric) 1/1

[00356] The treatment that the 18 patients with the PFS ≥1.3 received based on profiling is detailed in **Table 13**. As can be seen in that table for breast cancer patients, the treatment ranged from diethylstibesterol to nab paclitaxel + gemcitabine to doxorubicin. Treatments for patients with other tumor types are also detailed in **Table 13**. The table further shows a comparison of the drugs that the responding patients received versus the drugs that would have been suggested without molecular profiling and indicates which targets were used to suggest the therapies. Overall, 14 were treated with combinations and 4 were treated with single agents.

Table 13: Targets Noted in Patients' Tumors, Treatment Suggested on the Basis of These Results, and Treatment Investigator Would Use if No Target Was Identified (in patients with PFS ratio ≥ 1.3)

Location of Primary Tumor	**Targets Used to Suggest Treatment and Method Used**	**Treatment Suggested on Basis of Patient's Tumor Molecular Profiling**	**Treatment the Investigator Would Have Used if No Results From Molecular Profiling**
Breast	ESR1: I; *ESR1*: M	DES 5 mg TID	Investigational
Cholangiocarcinoma	EGFR: I; *TOP1*: M	CPT-11 350 mg/m^2 every 3 weeks; cetuximab 400 mg/m^2 day 1, 250 mg/m^2 every week	Investigational
Breast	SPARC: I; *SPARC*, *ERBB2*: M	NAB paclitaxel 260 mg/m^2 every 3 weeks; trastuzumab 6 mg/kg every 3 weeks	Docetaxel, trastuzumab
Eccrine sweat gland (right forearm)	c-KIT: I; *c-KIT*:M	Sunitinib 50 mg/d, 4 weeks on/2 weeks off	Best supportive care
Ovary	HER2/NEU, ER: I; *HER2/NEU*: M	Lapatinib 1,250 mg PO days 1-21; tamoxifen 20 mg PO	Bevacizumab
Colon/rectum	PDGFR, c-KIT: I I; *PDGFR, TOP1*: M	CPT-11 70 mg/m^2 weekly for 4 weeks on/2 weeks off; sorafenib 400 mg BID	Cetuximab
Breast	SPARC: I; *DCK*: M	NAB paclitaxel 90 mg/m^2 every 3 weeks;	Mitomycin

		gemcitabine 750 mg/m^2 days 1, 8, 15, every 3 weeks	
Breast	ER: I; *ER, TYMS*: M	Letrozole 2.5 mg daily; capecitabine 1,250 mg/m^2 BID, 2 weeks on/1 week off	Capecitabine
Malignant mesothelioma	MLH1, MLH2: I; *RRM2B*, *RRM1*, *RRM2*, *TOP2B*: M	Gemcitabine 1,000 mg/m2 days 1 and 8, every 3 weeks; etoposide 50 mg/m^2 3 days every 3 weeks	Gemcitabine
Breast	MSH2	Oxaliplatin 85 mg/m^2 every 2 weeks; fluorouracil (5FU) 1,200 mg/m^2 days 1 and 2, every 2 weeks; trastuzumab 4 mg/kg day 1, 2 mg/kg every week	Investigational
Non–small-cell lung cancer	EGFR: I; *EGFR*	Cetuximab 400 mg/m^2 day 1, 250 mg/m^2 every week; CPT-11 125 mg/m^2 weekly for 4 weeks on/2 weeks off	Vinorelbine
Colon/rectum	*MGMT*	Temozolomide 150 mg/m^2 for 5 days every 4 weeks; bevacizumab 5 mg/kg every 2 weeks	Capecitabine
Colon/rectum	PDGFR, c-KIT: I; *PDGFR*: *KDR*, *HIF1A*, *BRCA2*: M	Mitomycin 10 mg once every 4-6 weeks; sunitinib 37.5 mg/d, 4 weeks on/2 weeks off	Capecitabine
Breast	*DCK*, *DHFR*: M	Gemcitabine 1,000 mg/m^2 days 1 and 8 every 3 weeks; pemetrexed 500 mg/m^2 days 1 and 8, every 3 weeks	Best supportive care
Breast	TOP2A: I; *TOP2A*: M	Doxorubicin 50 mg/m^2 every 3 weeks	Vinorelbine
Colon/rectum	*MGMT*, *VEGFA*, *HIF1A*: M	Temozolomide 150 mg/m^2 for 5 days every 4 weeks; sorafenib 400 mg BID	Panitumumab
Breast	ESR1, PR: I; *ESR1, PR*: M	Exemestane 25 mg every day	Doxorubicin liposomal
GIST (stomach)	EGFR: I; *EGFR*, *RRM2*: M	Gemcitabine 1,000 mg/m^2 days 1, 8, and 15 every 4 weeks; cetuximab 400 mg/m^2 day 1, 250 mg/m^2 every week	None

* Abbreviations used in **Table 13**: I, immunohistochemistry; M, microarray; DES, diethylstilbestrol; CPT-11, irinotecan; TID, three times a day; NAB, nanoparticle albumin bound; PO, orally; BID, twice a day; GIST, GI stromal tumor.

[00357] *Secondary Endpoints*

[00358] The results for the secondary endpoint for this study are as follows. The frequency with which molecular profiling of a patients' tumor yielded a target in the 86 patients where molecular profiling was

attempted was 84/86 (98%). Broken down by methodology, 83/86 (97%) yielded a target by IHC/FISH and 81/86 (94%) yielding a target by microarray. RNA was tested for integrity by assessing the ratio of 28S to 18S ribosomal RNA on an Agilent BioAnalyzer. 83/86 (97%) specimens had ratios of 1 or greater and gave high intra-chip reproducibility ratios. This demonstrates that very good collection and shipment of patients' specimens throughout the United States and excellent technical results can be obtained.

[00359] By RECIST criteria in 66 patients, there was 1 complete response and 5 partial responses for an overall response rate of 10% (one CR in a patient with breast cancer and PRs in breast, ovarian, colorectal and NSCL cancer patients). Patients without progression at 4 months included 14 out of 66 or 21%.

[00360] In an exploratory analysis, a waterfall plot for all patients for maximum % change of the summed diameters of target lesions with respect to baseline diameters was generated. The patients who had progression and the patients who had some shrinkage of their tumor sometime during their course along with those partial responses by RECIST criteria is demonstrated in **FIG. 12**. There is some shrinkage of patient's tumors in over 47% of the patients (where 2 or more evaluations were completed).

[00361] *Other Analyses – Safety*

[00362] As far as safety analyses there were no treatment related deaths. There were nine treatment related serious adverse events including anemia (2 patients), neutropenia (2 patients), dehydration (1 patient), pancreatitis (1 patient), nausea (1 patient), vomiting (1 patient), and febrile neutropenia (1 patient). Only one patient (1.5%) was discontinued due to a treatment related adverse event of grade 2 fatigue.

[00363] *Other Analyses – Relationship between What the Clinician Caring for the Patient Would Have Selected versus What the Molecular Profiling Selected*

[00364] The relationship between what the clinician selected to treat the patient before knowing what molecular profiling results suggested for treatment was also examined. As detailed in **FIG. 13**, there is no pattern between the two. More specifically, no matches for the 18 patients with PFS ratio $\geq$1.3 were noted.

[00365] The overall survival for the 18 patients with a PFS ratio of $\geq$1.3 versus all 66 patients is shown in **FIG. 14**. This exploratory analysis was done to help determine if the PFS ratio had some clinical relevance. The overall survival for the 18 patients with the PFS ratio of $\geq$1.3 is 9.7 months versus 5 months for the whole population – log rank 0.026. This exploratory analysis indicates that the PFS ratio is correlated with the clinical parameter of survival.

[00366] ***Conclusions***

[00367] This prospective multi-center pilot study demonstrates: (a) the feasibility of measuring molecular targets in patients' tumors from 9 different centers across the US with good quality and sufficient tumor collection – and treat patients based on those results; (b) this molecular profiling approach gave a longer PFS for patients on a molecular profiling suggested regimen than on the regimen they had just progressed on for 27% of the patients (confidence interval 17-38%) p = 0.007; and (c) this is a promising result demonstrating use and benefits of molecular profiling.

[00368] The results also demonstrate that patients with refractory cancer can commonly have simple targets (such as ER) for which therapies are available and can be beneficial to them. Molecular profiling for patients who have exhausted other therapies and who are perhaps candidates for phase I or II trials could have this molecular profiling performed.

Example 5: Molecular Profiling System

[00369] Molecular profiling is performed to determine a treatment for a disease, typically a cancer. Using a molecular profiling approach, molecular characteristics of the disease itself are assessed to determine a candidate treatment. Thus, this approach provides the ability to select treatments without regard to the anatomical origin of the diseased tissue, or other "one-size-fits-all" approaches that do not take into account personalized characteristics of a particular patient's affliction. The profiling comprises determining gene and gene product expression levels, gene copy number and mutation analysis. Treatments are identified that are indicated to be effective against diseased cells that overexpress certain genes or gene products, underexpress certain genes or gene products, carry certain chromosomal aberrations or mutations in certain genes, or any other measureable cellular alterations as compared to non-diseased cells. Because molecular profiling is not limited to choosing amongst therapeutics intended to treat specific diseases, the system has the power to take advantage of any useful technique to measure any biological characteristic that can be linked to a therapeutic efficacy. The end result allows caregivers to expand the range of therapies available to treat patients, thereby providing the potential for longer life span and/or quality of life than traditional "one-size-fits-all" approaches to selecting treatment regimens.

[00370] A molecular profiling system has several individual components to measure expression levels, chromosomal aberrations and mutations. The components are shown in **FIG. 15**. These include immunohistochemistry assays (IHC) on formalin fixed paraffin embedded (FFPE) cancer tissue. To perform IHC on a sample, a paraffin embedded block with a large section of tumor (at least 20% viable neoplasm) from the procedure which is preferred. For any tumor, IHC is run for 18 target genes comprising druggable or drug resistant targets. IHC can be performed on additional genes depending on disease characteristics, e.g., tumor origin and progression. In addition to IHC, gene expression arrays, such as the Agilent 44K chip (Agilent Technologies, Inc., Santa Clara, CA). This system is capable of determining the relative expression level of roughly 44,000 different sequences through RT-PCR from RNA extracted from fresh frozen tissue. The expression of 80 druggable or drug resistant targets is examined in further detail. Because of the practicalities involved in obtaining fresh frozen tissue, only a portion of samples with sufficient quantity and quality of mRNA are analyzed using microarray analysis. The system also assesses gene copy number and/or other chromosomal abnormalities for a number of genes using FISH (fluorescence in situ hybridization). Finally, mutation analysis is done by DNA sequencing for a several specific mutations. All of this data is stored for each patient case. Microarray results IHC, FISH and DNA sequencing analysis for a number of genes that have been shown to impact therapeutic options are used to generate a final patient report. The report can include a prioritized list of druggable targets and their associated therapies. The report is explained by a practicing oncologist. Once the data are reported, the final decisions rest with the treating physician. Based on this approach, the treating physician has information on therapies that might not otherwise have been considered based on the lineage of the disease.

Example 6: Molecular Profiling System and Report

[00371] A system has several individual components including a gene expression array using the Illumina Whole Genome DASL Assay. The Illumina Whole Genome DASL assay (Illumina Inc., San Diego, CA) offers a method to simultaneously profile over 24,000 transcripts from minimal RNA input, from both fresh frozen

(FF) and formalin-fixed paraffin embedded (FFPE) tissue sources, in a high throughput fashion. The analysis makes use of the Whole-Genome DASL Assay with UDG (Illumina, cat#DA-903-1024/DA-903-1096), the Illumina Hybridization Oven, and the Illumina iScan System. The system is used according to the manufacturer's instructions. Genes are identified as overexpressed, underexpressed or no change in expression as compared to a threshold using desired statistical measures.

[00372] In addition to this gene expression array, the system also performs a subset of immunohistochemistry assays on formalin fixed paraffin embedded (FFPE) cancer tissue. Gene copy number is determined for a number of genes via FISH (fluorescence in situ hybridization) and mutation analysis is done by DNA sequencing for a several specific mutations. All of this data is stored for each patient case. Data is reported from the microarray, IHC, FISH and DNA sequencing analysis. All laboratory experiments are performed according to Standard Operating Procedures (SOPs).

[00373] DNA for mutation analysis is extracted from formalin-fixed paraffin-embedded (FFPE) tissues after macrodissection of the fixed slides in an area that % tumor nuclei ≥ 10% as determined by a pathologist. Extracted DNA is only used for mutation analysis if % tumor nuclei ≥ 10%. DNA is extracted using the QIAamp DNA FFPE Tissue kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). DNA can also be extracted using the QuickExtract™ FFPE DNA Extraction Kit according to the manufacturer's instructions (Epicentre Biotechnologies, Madison, WI). The BRAF Mutector I BRAF Kit (TrimGen, cat#MH1001-04) is used to detect BRAF mutations (TrimGen Corporation, Sparks, MD). The DxS KRAS Mutation Test Kit (DxS, #KR-03) is used to detect KRAS mutations (QIAGEN Inc., Valencia, CA). BRAF and KRAS sequencing of amplified DNA is performed using Applied Biosystems' BigDye® Terminator V1.1 chemistry (Life Technologies Corporation, Carlsbad, CA).

[00374] IHC is performed according to standard protocols. IHC detection systems vary by marker and include Dako's Autostainer Plus (Dako North America, Inc., Carpinteria, CA), Ventana Medical Systems Benchmark® XT (Ventana Medical Systems, Tucson, AZ), and the Leica/Vision Biosystems Bond System (Leica Microsystems Inc., Bannockburn, IL). All systems are operated according to the manufacturers' instructions. American Society of Clinical Oncology (ASCO) and College of American Pathologist (CAP) standards are followed for ER, PR, and HER2 testing. ER, PR and HER2 as well as Ki-67, p53, and E-cad IHCs analyzed by the ACIS® (Automated Cellular Imaging System). The ACIS system comprises a microscope that scans the slides and constructs an image of the entire tissue section. Ten areas of tumor are analyzed for percentage positive cells and staining intensity within the selected fields.

[00375] FISH is performed on formalin-fixed paraffin-embedded (FFPE) tissue. FFPE tissue slides for FISH must be Hematoxylin and Eosin (H&E) stained and given to a pathologist for evaluation. Pathologists will mark areas of tumor for FISH analysis. The pathologist report shows whether tumor is present and sufficient enough to perform a complete analysis. FISH is performed using the Abbott Molecular VP2000 according to the manufacturer's instructions (Abbott Laboratories, Des Plaines, IA).

[00376] An illustrative report generated by the system in shown in **FIGS. 16A-16N**. **FIG. 16A** shows that the patient had a primary tumor in the breast determined to be HER2+, and provides a Summary of candidate therapeutic agents associated with beneficial or not for treating the tumor based on molecular profiling results. **FIG. 16B** illustrates a more detailed Summary listing for each agent associated with benefit, including the informative biomarkers and experimental methods used to assess those biomarkers. **FIG. 16C** illustrates a more

detailed Summary listing for each agent associated with lack of benefit, including the informative biomarkers and experimental methods used to assess those biomarkers. **FIG. 16D** and **FIG. 16E** present the results of IHC analysis. **FIG. 16F** and **FIG. 16G** present the results of DNA microarray analysis, wherein results for informative biomarkers are shown in **FIG. 16F** whereas the non-informative biomarkers are shown in **FIG. 16G**. “Non-informative” indicates that the data obtained for the patient sample or control sample were not of sufficiently high quality to confidently evaluate the expression level of those RNA transcripts. FISH analysis is presented in **FIG. 16H** and mutational analysis is presented in **FIG. 16I**. Mutational analysis included direct sequence analysis of exon 9 of PIK3CA. **FIG. 16J** and **FIG. 16K** present a summary description of the relevant biomarkers. **FIG. 16L** and **FIG. 16M** present a summary description of literature supporting the candidate therapeutics linked to the informative biomarkers with a rating for the level of evidence attached to each publication. **FIG. 16N** is a chart depicting the codes for level of evidence.

Example 7: Detection of SPARC and HSP90

[00377] A 25 year-old woman was presented with a solitary enlarged right axillary lymph node. Prior medical history includes hyperreninemic hypertension, without an identified pathologic lesion, requiring angiotensin receptor blockade medication one year preceding the cancer diagnosis, and a sinus infection requiring antibiotic therapy immediately preceding the cancer presentation. The patient initially noted a non-tender swelling under her right axilla and sought further medical evaluation. Upon completion of antibiotics for the sinus infection, the patient underwent an ultrasound and excisional biopsy of the enlarged right axillary lymph node with clear margins. Patient’s family history negative for blood disorders or malignancy and she denied tobacco or alcohol use.

[00378] H&E and IHC staining of biopsy showed strongly positive signal for S100 in majority of cells, negative signal for MelanA, and moderately positive signal for smooth muscle actin in majority of cells. Bone marrow biopsy revealed normocellular marrow with adequate trilineage hematopoiesis, and markedly decreased iron staining; normal karyotype, 46 XX. Flow cytometry revealed no monoclonality, evidence of lymphoma or leukemia. The final pathologic diagnosis was interdigitating dendritic cell sarcoma (IDCS) after independent confirmation at a reference pathology laboratory.

[00379] [18F]-2-fluoro-deoxy-D-glucose (FDG) computed tomography (PET/CT) revealed low uptake of FDG tracer in right axilla in area of excision. No adenopathy was visualized. The patient was followed conservatively without receiving adjuvant chemotherapy or radiotherapy Follow-up PET/CT scans at 4, 7, 11, and 18 months showed no evidence of disease recurrence and physical exam was stable and remained unremarkable. She is now followed conservatively on an annual basis with physical examination and imaging.

[00380] IHC molecular characterization for treatment targets was performed according to the methods of the invention. See **FIG. 2**. The tumor was positive for SPARC and HSP90, but negative for PDGFR, MSH1, MSH2, c-kit, Her2/Neu, P-glycoprotein, ER, PR, Androgen Receptor, CD25, and CD52.

Example 8: Diagnosis of a Sarcoma

[00381] A sample is obtained from a subject having a suspected tumor by physical examination. The sample is sectioned into thin slices of tissue and embedded in paraffin. Each paraffin section is mounted onto a slide. Slides are incubated with anti-SPARC antibody and/or anti-HSP90 antibody. For visualization and detection of

antibodies bound to SPARC or HSP90, secondary antibodies conjugated with fluorescent dyes are added to each slide. In a double staining of anti-SPARC and anti-HSP90 antibodies, secondary antibodies of fluorescent dyes with distinguishable spectra are used. The slides are visualized by providing excitation beams to fluorescent dyes. Images of fluorescent signals are taken by fluorescent microscopes. To prepare a comparable control sample, the procedure is repeated with samples obtained from another subject deemed clinically normal or free of observable tumor. The intensity of fluorescent signals obtained from tumor sample is compared to that of control sample. Greater intensity in the suspected tumor sample as compared to the control indicates a diagnosis of sarcoma.

Example 8: Prognosis of a Sarcoma

[00382] A sample is obtained from a subject having a sarcoma. The sample is sectioned into thin slices of tissue and embedded in paraffin. Each paraffin section is mounted onto a slide. Slides are incubated with anti-SPARC antibody and/or anti-HSP90 antibody. For visualization and detection of antibodies bound to SPARC or HSP90, secondary antibodies conjugated with fluorescent dyes are added to each slide. In a double staining of anti-SPARC and anti-HSP90 antibodies, secondary antibodies of fluorescent dyes with distinguishable spectra are used. The slides are visualized by providing excitation beams to fluorescent dyes. Images of fluorescent signals are taken by fluorescent microscopes. To prepare comparable control samples, the procedure is repeated with samples obtained from other subject deemed clinically normal or free of observable tumor or with varying degrees and stages of the sarcoma. The intensity of fluorescent signals obtained from tumor sample is compared to that of the control samples. The intensity in the suspected tumor sample is correlated with the intensity in the control samples, wherein a higher correlation between samples indicates a more similar prognosis.

Example 8: Treatment Monitoring of a Sarcoma

[00383] A sample is obtained from a subject having a sarcoma being treated with anti-mitotic agents and/or HSP90 inhibitors. The sample is sectioned into thin slices of tissue and embedded in paraffin. Each paraffin section is mounted onto a slide. Slides are incubated with anti-SPARC antibody and/or anti-HSP90 antibody. For visualization and detection of antibodies bound to SPARC or HSP90, secondary antibodies conjugated with fluorescent dyes are added to each slide. In a double staining of anti-SPARC and anti-HSP90 antibodies, secondary antibodies of fluorescent dyes with distinguishable spectra are used. The slides are visualized by providing excitation beams to fluorescent dyes. Images of fluorescent signals are taken by fluorescent microscopes. Similar samples are taken over the time course of treatment. The intensity of fluorescent signals obtained from the sample is observed over time. Increasing intensity over time indicates that the subject is not responding to the treatments. Decreasing intensity over time indicates that the subject is responding to the treatments.

Example 10: Detecting SPARC and HSP90 in a Renal Cell Carcinoma

[00384] A sample is obtained from a cancerous region of human renal cell carcinoma. The sample is sectioned into thin slices of tissue and embedded in paraffin. Each paraffin section is mounted onto a slide. Each slide is incubated with either anti-SPARC antibody or anti-HSP90 antibody, or both. To visualized antibodies bound to SPARC or HSP90, secondary antibodies conjugated with fluorescent dyes are added to each slide. In a double

staining of anti-SPARC and anti-HSP90 antibodies, secondary antibodies of fluorescent dyes with distinguishable spectra are used. The slides are visualized by providing excitation beams to fluorescent dyes. Images of fluorescent signals are taken by fluorescent microscopes. To prepare a comparable control sample, the procedure is repeated with samples obtained from a noncancerous region. The intensity of fluorescent signals obtained from tumor sample is compared to that of control sample.

[00385] If the analysis reveals that SPARC and/or HSP90 are overexpressed in the sample, a candidate agent can be selected that is associated with SPARC and/or HSP90 as appropriate, using the methods described herein.

[00386] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of selecting a candidate treatment for a malignancy in a subject comprising:
   (a) obtaining a sample of the malignancy;
   (b) detecting a level of SPARC and HSP90 in the sample; and
   (c) selecting a treatment associated with SPARC and HSP90 if the sample has an elevated level of SPARC and HSP90 as compared to a reference.
2. The method of claim **1**, wherein the reference is from a non-malignant sample.
3. The method of claim **1**, wherein the reference is from the subject.
4. The method of claim **1**, wherein the level of SPARC and HSP90 in step (b) is detected using one or more of IHC, FISH, PCR, microarray and sequencing.
5. The method of claim **4**, wherein the microarray analysis comprises using a low density microarray, an expression microarray, a comparative genomic hybridization (CGH) microarray, a single nucleotide polymorphism (SNP) microarray, a proteomic array or an antibody array.
6. The method of claim **4**, wherein the microarray analysis comprises identifying whether a gene is significantly upregulated or downregulated relative to a reference.
7. The method of claim **6**, wherein the significance is determined at a p-value of less than or equal to 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001.
8. The method of claim **7**, wherein the p-value is corrected for multiple comparisons.
9. The method of claim **8**, wherein the correction for multiple comparisons comprises Bonneferoni's correction, a false discovery rate, or a modification of any thereof.
10. The method of claim **4**, wherein the IHC analysis comprises determining whether 30% or more of the sample is +2 or greater in staining intensity.
11. The method of claim **10**, wherein the SPARC and HSP90 are elevated if 30% or more of the sample is +2 or greater in staining intensity for SPARC and HSP90 as determined by the IHC analysis.
12. The method of claim **1**, wherein a prioritized list of candidate treatments is identified.
13. The method of claim **12**, wherein prioritizing comprises ordering the treatments from higher priority to lower priority according to treatments based on either IHC or FISH analysis of the SPARC and HSP90 and microarray analysis; treatments based on IHC analysis of the SPARC and HSP90 but not microarray analysis; and treatments based on microarray analysis of the SPARC and HSP90 but not IHC analysis.
14. The method of claim **1**, wherein the treatment comprises one or more therapeutic agent.
15. The method of claim **14**, wherein the one or more therapeutic agent comprises one or more mitotic inhibitor.
16. The method of claim **15**, wherein the one or more mitotic inhibitor comprises a taxane, a vinca alkaloid, or a combination thereof.
17. The method of claim **16**, wherein the taxane comprises paclitaxel, nab-paclitaxel, paclitaxel bound to albumin, or docetaxel.
18. The method of claim **16**, wherein the vinca alkaloid comprises vincristine, vinblastine, vindesine or vinorelbine.

19. The method of claim **14**, wherein the one or more therapeutic agent comprises one or more HSP90 inhibitor.

20. The method of claim **19**, wherein the one or more HSP90 inhibitor comprises geldanamycin, 17-*N*-Allylamino-17-demethoxygeldanamycin (17-AAG), 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), IPI-504 (retaspimycin), BIIB021 (CNF2024), BIIB028, SNX-5422, Ganetespib STA-9090, AUY922, AT13387, cisplatin, herbimycin, radicicol, novobiocin, coumermycin A1, clorobiocin, epigallocatechin gallate (EGCG), taxol, pochonin, derrubone, gedunin, celastrol, or a derivative of any thereof.

21. The method of claim **1**, further comprising molecular profiling of one or more of ABCC1, ABCG2, ACE2, ADA, ADH1C, ADH4, AGT, AR, AREG, ASNS, BCL2, BCRP, BDCA1, beta III tubulin, BIRC5, B-RAF, BRCA1, BRCA2, CA2, caveolin, CD20, CD25, CD33, CD52, CDA, CDKN2A, CDKN1A, CDKN1B, CDK2, CDW52, CES2, CK 14, CK 17, CK 5/6, c-KIT, c-Met, c-Myc, COX-2, Cyclin D1, DCK, DHFR, DNMT1, DNMT3A, DNMT3B, E-Cadherin, ECGF1, EGFR, EML4-ALK fusion, EPHA2, Epiregulin, ER, ERBR2, ERCC1, ERCC3, EREG, ESR1, FLT1, folate receptor, FOLR1, FOLR2, FSHB, FSHPRH1, FSHR, FYN, GART, GNRH1, GNRHR1, GSTP1, HCK, HDAC1, hENT-1, Her2/Neu, HGF, HIF1A, HIG1, HSPCA, HSP90AA1, IGF-1R, IGFRBP, IGFRBP3, IGFRBP4, IGFRBP5, IL13RA1, IL2RA, KDR, Ki67, KIT, K-RAS, LCK, LTB, Lymphotoxin Beta Receptor, LYN, MET, MGMT, MLH1, MMR, MRP1, MS4A1, MSH2, MSH5, Myc, NFKB1, NFKB2, NFKBIA, ODC1, OGFR, p16, p21, p27, p53, p95, PARP-1, PDGFC, PDGFR, PDGFRA, PDGFRB, PGP, PGR, PI3K, POLA, POLA1, PPARG, PPARGC1, PR, PTEN, PTGS2, RAF1, RARA, RRM1, RRM2, RRM2B, RXRB, RXRG, SRC, SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Survivin, TK1, TLE3, TNF, TOP1, TOP2A, TOP2B, TS, TXN, TXNRD1, TYMS, VDR, VEGF, VEGFA, VEGFC, VHL, YES1, and ZAP70.

22. The method of claim **1**, wherein the subject has been previously treated with the candidate treatment.

23. The method of claim **1**, wherein the subject has not previously been treated with one or more candidate therapeutic agents.

24. The method of claim **1**, wherein the malignancy comprises a metastatic malignancy.

25. The method of claim **1**, wherein the malignancy comprises a recurrent malignancy.

26. The method of claim **1**, wherein the malignancy is refractory to a prior treatment.

27. The method of claim **26**, wherein the prior treatment comprises the standard of care for the malignancy.

28. The method of claim **1**, wherein the malignancy comprises a malignancy of a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, or an adipose tissue.

29. The method of claim **1**, wherein the malignancy comprises a carcinoma or sarcoma.

30. The method of claim **1**, wherein the malignancy comprises a renal cell carcinoma.

31. The method of claim **1**, wherein the malignancy comprises an interdigitating dendritic cell sarcoma.

32. The method of claim **1**, wherein the malignancy comprises an acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related cancer; AIDS-related lymphoma; anal cancer; appendix cancer; astrocytomas; atypical teratoid/rhabdoid tumor; basal cell carcinoma; bladder cancer; brain stem glioma; brain tumor, brain stem glioma, central nervous system atypical teratoid/rhabdoid tumor, central nervous system embryonal tumors, astrocytomas, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, supratentorial

primitive neuroectodermal tumors and pineoblastoma; breast cancer; bronchial tumors; Burkitt lymphoma; cancer of unknown primary site (CUP); carcinoid tumor; carcinoma of unknown primary site; central nervous system atypical teratoid/rhabdoid tumor; central nervous system embryonal tumors; cervical cancer; childhood cancers; chordoma; chronic lymphocytic leukemia; chronic myelogenous leukemia; chronic myeloproliferative disorders; colon cancer; colorectal cancer; craniopharyngioma; cutaneous T-cell lymphoma; endocrine pancreas islet cell tumors; endometrial cancer; ependymoblastoma; ependymoma; esophageal cancer; esthesioneuroblastoma; Ewing sarcoma; extracranial germ cell tumor; extragonadal germ cell tumor; extrahepatic bile duct cancer; gallbladder cancer; gastric (stomach) cancer; gastrointestinal carcinoid tumor; gastrointestinal stromal cell tumor; gastrointestinal stromal tumor (GIST); gestational trophoblastic tumor; glioma; hairy cell leukemia; head and neck cancer; heart cancer; Hodgkin lymphoma; hypopharyngeal cancer; intraocular melanoma; islet cell tumors; Kaposi sarcoma; kidney cancer; Langerhans cell histiocytosis; laryngeal cancer; lip cancer; liver cancer; malignant fibrous histiocytoma bone cancer; medulloblastoma; medulloepithelioma; melanoma; Merkel cell carcinoma; Merkel cell skin carcinoma; mesothelioma; metastatic squamous neck cancer with occult primary; mouth cancer; multiple endocrine neoplasia syndromes; multiple myeloma; multiple myeloma/plasma cell neoplasm; mycosis fungoides; myelodysplastic syndromes; myeloproliferative neoplasms; nasal cavity cancer; nasopharyngeal cancer; neuroblastoma; Non-Hodgkin lymphoma; nonmelanoma skin cancer; non-small cell lung cancer; oral cancer; oral cavity cancer; oropharyngeal cancer; osteosarcoma; other brain and spinal cord tumors; ovarian cancer; ovarian epithelial cancer; ovarian germ cell tumor; ovarian low malignant potential tumor; pancreatic cancer; papillomatosis; paranasal sinus cancer; parathyroid cancer; pelvic cancer; penile cancer; pharyngeal cancer; pineal parenchymal tumors of intermediate differentiation; pineoblastoma; pituitary tumor; plasma cell neoplasm/multiple myeloma; pleuropulmonary blastoma; primary central nervous system (CNS) lymphoma; primary hepatocellular liver cancer; prostate cancer; rectal cancer; renal cancer; renal cell (kidney) cancer; renal cell cancer; respiratory tract cancer; retinoblastoma; rhabdomyosarcoma; salivary gland cancer; Sézary syndrome; small cell lung cancer; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; squamous neck cancer; stomach (gastric) cancer; supratentorial primitive neuroectodermal tumors; T-cell lymphoma; testicular cancer; throat cancer; thymic carcinoma; thymoma; thyroid cancer; transitional cell cancer; transitional cell cancer of the renal pelvis and ureter; trophoblastic tumor; ureter cancer; urethral cancer; uterine cancer; uterine sarcoma; vaginal cancer; vulvar cancer; Waldenström macroglobulinemia; a Wilm's tumor; or a cancer of unknown primary (CUP).

33. The method of claim **1**, wherein progression free survival (PFS) or disease free survival (DFS) for the subject is extended.

34. The method of claim **1**, wherein the subject's lifespan is extended by selection of the candidate treatment.

35. A method for diagnosing or prognosing a malignancy in a subject, comprising:

(a) obtaining a sample from the subject;

(b) detecting whether the sample has an elevated level of SPARC and HSP90 as compared to a reference; and

(c) diagnosing or prognosing the malignancy if the sample has an elevated level of SPARC.

36. The method of claim **35**, wherein the level of SPARC and HSP90 in step (b) is elevated if least 30% of the cells in the sample are positive for SPARC and HSP90.

37. The method of claim **35**, wherein the diagnosis is the presense of malignancy if the levels of SPARC and HSP90 are elevated.

38. The method of claim **35**, wherein greater elevation of the levels of SPARC and HSP90 indicate a worse prognosis.

39. The method of claim **35**, wherein the malignancy comprises a malignancy of a lymph node, a bone marrow, a lung, an ovary, a breast, a head, a neck, a pancreas, a colon, a melanocyte, an adrenal cortex, or an adipose tissue.

40. The method of claim **35**, wherein the malignancy comprises a carcinoma or sarcoma.

41. The method of claim **35**, wherein the malignancy comprises a renal cell carcinoma.

42. The method of claim **35**, wherein the malignancy comprises an interdigitating dendritic cell sarcoma.

43. The method of any preceding claim, wherein the method is performed in vitro.

44. Use of a reagent for carrying out the method of any preceding claim.

45. A method of detecting an elevated level of SPARC or HSP90 in a biological tissue comprising the steps of:

(a) providing a slide having a fixed sample contained thereon;
(b) deparaffinizing and rehydrating or further processing the sample for histochemistry;
(c) rinsing the sample with at least one of a buffer and another aqueous liquid;
(d) covering the sample with a peroxide or other oxidative agent for approximately 1 to 60 minutes;
(e) rinsing the tissue with at least one of a buffer and another aqueous liquid;
(f) covering the sample with at least one primary SPARC antibody or at least one primary HSP90 antibody diluted in a Tris based diluent, a negative control reagent, or another suitable carrier solution for approximately 5 to 120 minutes without prior or subsequent application of a blocking agent to the sample;
(g) rinsing the sample with at least one of a buffer and another aqueous liquid;
(h) covering the sample with at least one secondary detection reagent;
(i) rinsing the sample with at least one of a buffer and another aqueous liquid;
(j) covering the sample with a horseradish peroxidase conjugate, an enzymatic agent, or other catalytic agent;
(k) rinsing the sample with at least one of a buffer and another aqueous liquid;
(l) covering the sample with a first volume of at least one of a 3, 3'-diaminobenzidine containing solution and a chromogen containing solution for approximately 1 to 60 minutes;
(m) covering the sample with a second volume of at least one of a 3, 3'-diaminobenzidine containing solution and a chromogen containing solution for approximately 1 to 60 minutes;
(n) rinsing the sample with at least one of water and another aqueous liquid;
(o) covering the sample with a hematoxylin counterstain for approximately 2 minutes;
(p) rinsing the sample with at least one of a buffer and another aqueous liquid;
(q) repeatedly dipping the sample and the slide in distilled water until the slide is clear;
(r) dehydrating the sample;
(s) applying a cover slip over the sample contained on the slide; and

(t) detecting an elevated level of SPARC or HSP90 by examining the sample under a microscope and comparing the sample to a control.

46. The method of claim **45** wherein the step of covering the sample with at least one primary SPARC antibody or at least one primary HSP90 antibody comprises the step of covering the sample with one or more monoclonal antibodies and the step of covering the sample with at least one secondary detection reagent comprises the step of covering the sample with one or more monoclonal antibody probe reagents.

47. The method of claim **46** wherein the step of covering the tissue with one or more monoclonal antibody probe reagents comprises the step of covering the tissue with a mouse monoclonal antibody probe reagent for approximately 20 minutes and the step of covering the sample with a horseradish peroxidase conjugate, and enzymatic agent, or other catalytic agent comprises the step of covering the tissue with a horseradish peroxidase for approximately 20 minutes.

48. The method of claim **45** wherein the step of covering the tissue with at least one primary SPARC antibody or at least one primary HSP90 antibody comprises the step of covering the sample with one or more polyclonal antibodies and the step of covering the sample with at least one secondary detection reagent comprises the step of covering the sample with one or more polyclonal antibody probe reagents.

49. The method of claim **48** wherein the step of covering the sample with one or more polyclonal antibody probe reagents comprises the step of covering the sample with a goat anti-rat immunoglobulin G reagent for approximately 15 minutes and the step of covering the sample with a horseradish peroxidase conjugate, and enzymatic agent, or other catalytic agent comprises the step of covering the sample with a streptavidin horseradish peroxidase reagent for approximately 15 minutes.

50. The method of claim **45** wherein the step of detecting an elevated level of SPARC or HSP90 comprises the step of examining the sample under a microscope and comparing the sample to at least one of a negative control for SPARC or HSP90 and a positive control for SPARC or HSP90.

51. The method of claim **50** further comprising the step of assigning a value to the intensity of staining for a sample that has an elevated level of SPARC or HSP90.

52. The method of claim **51** further comprising the step of assigning an overall score to a sample that has an elevated level of SPARC or HSP90 wherein the overall score is based on the intensity of staining and the percent of cells stained in the sample.

53. The method of claim **52** wherein the overall score comprises at least one of a negative score, a moderately positive score, and a strongly positive score.

54. The method of claim **45** wherein at least steps c) through p) are automated such that they do not require manual handling of the samples and slides.

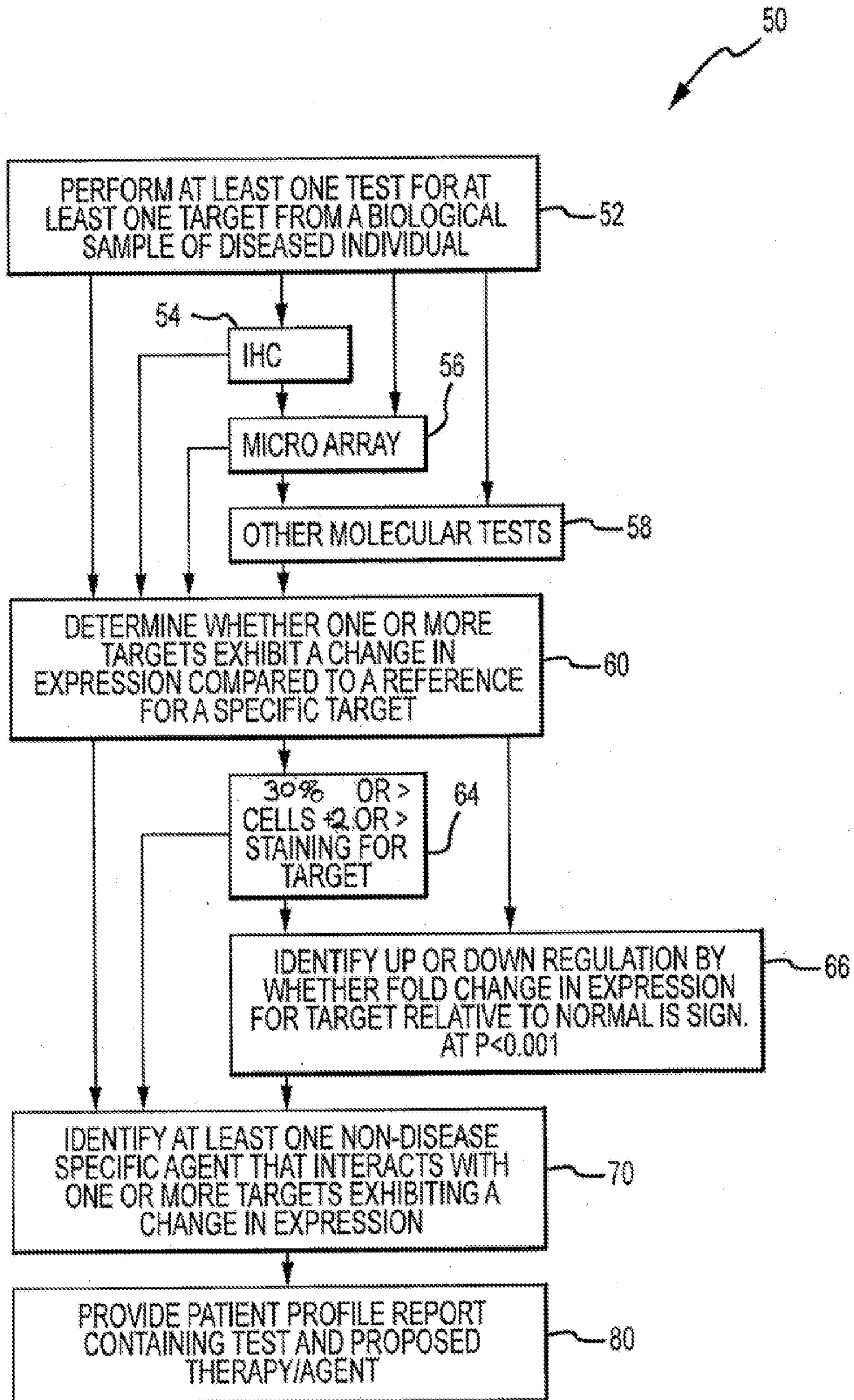


Figure 1

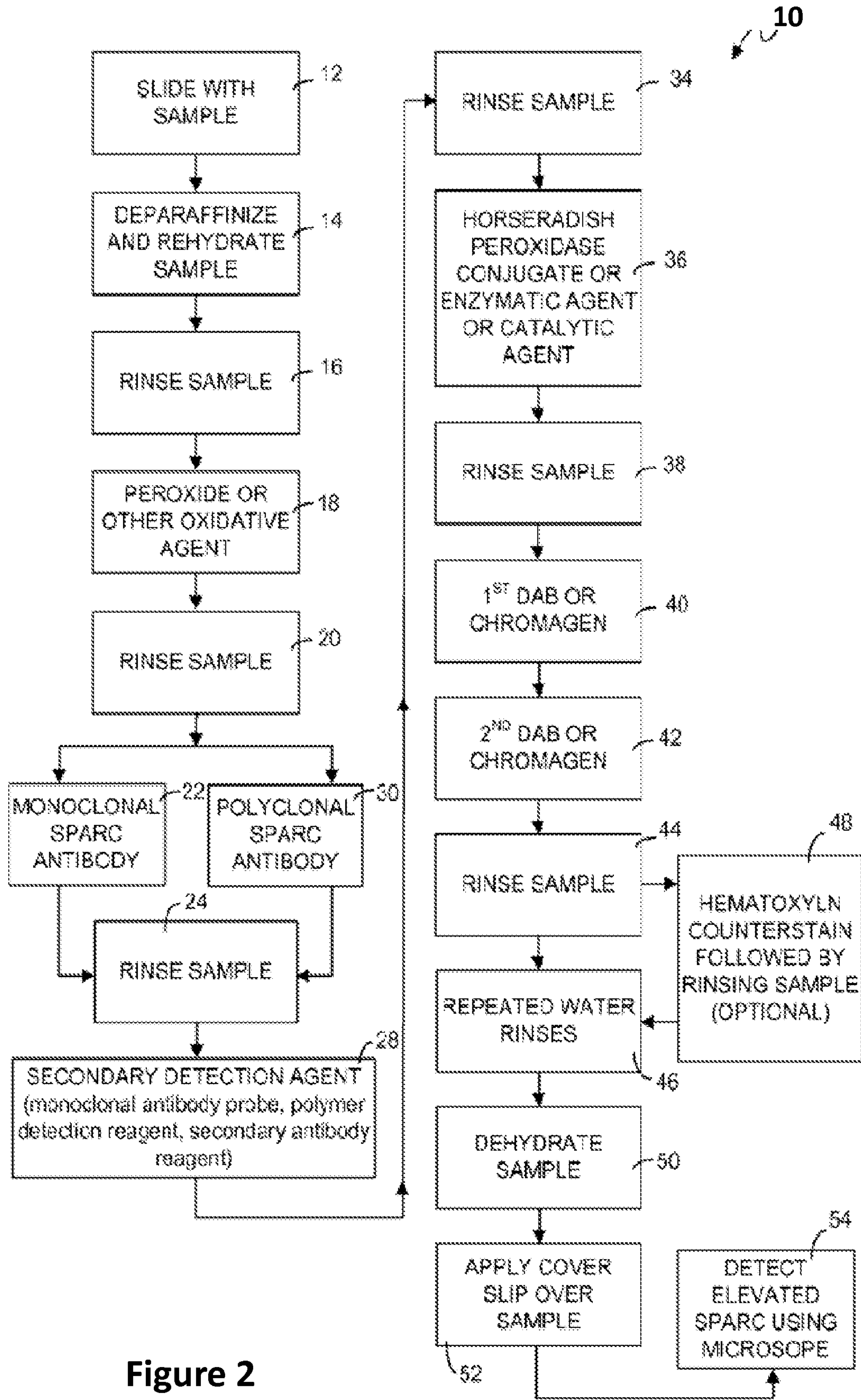
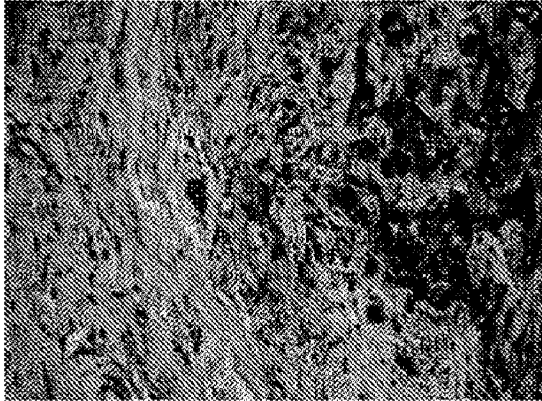
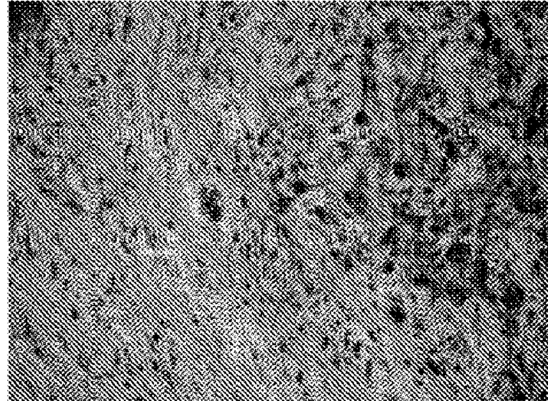


Figure 2

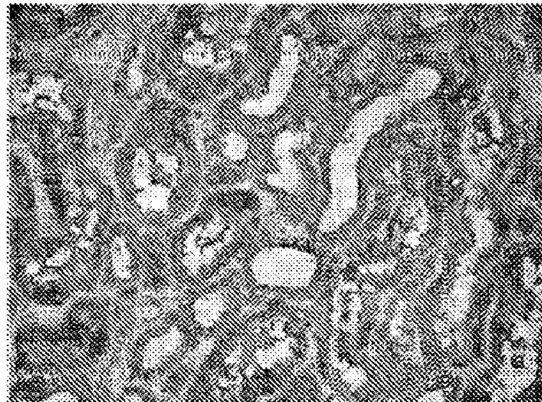
SPARC Polyclonal (Patient Sample)



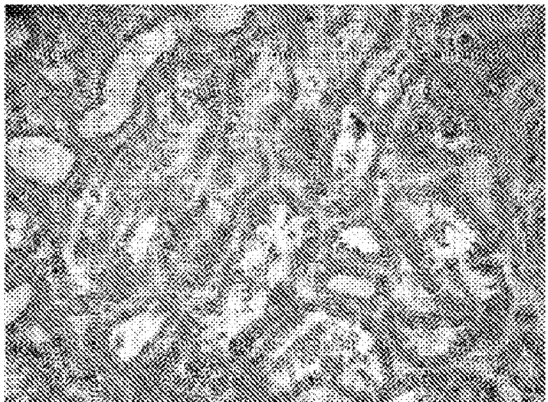
SPARC Monoclonal (Patient Sample)



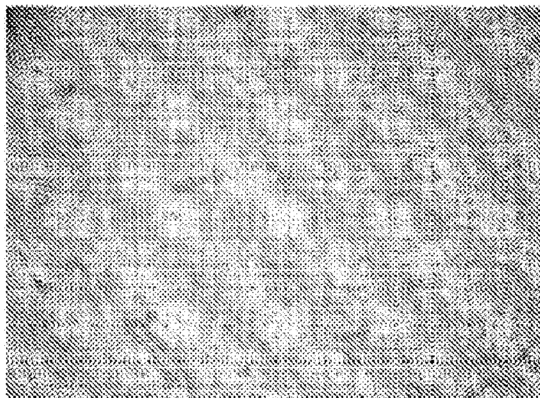
SPARC Polyclonal (Positive Control)



SPARC Monoclonal (Positive Control)



SPARC Polyclonal (Negative Control)



SPARC Monoclonal (Negative Control)

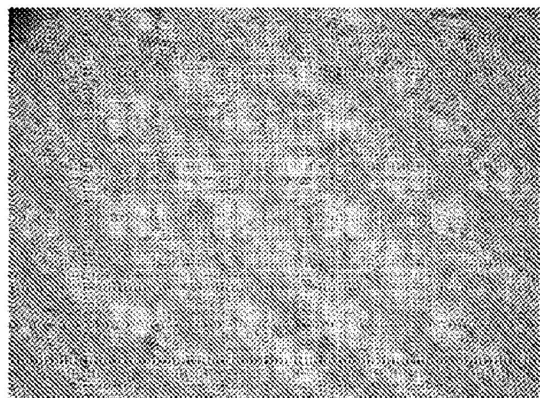


Figure 3

Blue=Scottsdale Health	Monoclonal				Polyclonal			
	Tumor		Stroma		Tumor		Stroma	
	Intensity	%	Intensity	%	Intensity	%	Intensity	%
Case No								
MP-TN06-06227	0	0	0	0	3*	60*	2*	20*
MP-TN06-06230	2	50	2	75	2	20	3	75
MP-TN06-06295	3	90	2	60	3	80	3	80
MP-TN06-06300	2	25	1	30	2	30	2	50
MP-TN06-06308	2	75	2	10	2	40	3	75
MP-TN06-06328	3	90	3	90	1	25	3	75
MP-TN06-06334	2	60	1	40	2	40	3	75
MP-TN06-06445	2	75	2	50	2	80	3	90
MP-TN06-06457	2	30	2	60	2	75	2	80
MP-TN07-07023	2	75	3	80	3	75	3	80
MP-TN07-07037	2	30	2	30	2	60	2	80
MP-TN07-07038	2	90	1	60	2	30	3	80
MP-TN07-07083	0	0	0	0	2*	30*	2*	50*
MP-TN07-07089	1	5	1	5	2*	95*	2*	80*
MP-TN07-07151	2	30	1	10	2	30	2	40
MP-TN07-07159	1	20	2	50	2	30	2	90
MP-TN07-07164	2	30	2	60	2	80	2	75
MP-TN07-07195	2	50	2	75	1	20	2	75
MP-TN07-07228	2	50	2	75	0	0	2	75
MP-TN07-07287	1	5	2	5	2*	80*	2*	75*
MP-TN07-07367	2	10	2	10	2	10	2*	60*
MP-TN08-08019	2	80	2	90	2	40	3	90
MP-TN08-08058	2	40	2	60	2	75	3	80
MP-TN08-08148	2	80	2	50	2	30	1	40
MP-TN08-08153	2	75	2	50	2	20	2	50
MP-TN08-08167	2	30	2	50	1	20	3	40
MP-TN08-08177	2*	90*	2*	60*	2	10	2	20

Figure 4A

MP-TN08-08191		1	30	2	20	2*	30*	2*	50*
MP-TN08-08193		2	50	2	75	1	30	3	75
MP-TN08-08209		2	50	2	40	3	60	2	50
MP-TN08-08210		2	50	2	40	1	80	3	80
MP-TN08-08236		2	50	2	30	1	5	3	60
MP-TN08-08250		2	80	2	80	3	30	3	75
MP-TN08-08253		2	75	3	90	0	0	2	75
MP-TN08-08256		2	20	2	20	2	50	2	75
MP-TN08-08388		2	75	2	60	3	75	3	90
MP-TN08-08392		2	40	2	50	1	5	3	90
MP-TN08-08395		0	0	0	0	2*	30*	2*	40*
MP-TN08-08425		2	50	2	40	2	5	2	75
MP-TN08-08459		2	75	2	40	2	20	3	60
MP-TN08-08466		2	60	2	80	1	10	2	40
MP-TN08-08467		3	90	2	80	2	50	3	80
MP-TN08-08471		2	90	2	90	2	90	3	90
MP-TN08-08476		3	95	3	95	0	0	2	75
MP-TN08-08477		2	90	2	40	2	75	3	75
MP-TN08-08480		2	75	2	50	1	5	3	40
MP-TNB07-07012		2	40	2	80	1	60	3	80
MP-TNB07-07207		2	30	1	10	2	60	2	30
MP-TNB07-07407		1	1	2	10	2*	60*	1*	75*
MP-TNB07-07409		2	30	1	20	2	40	2	75
MP-TNB08-08071		3	75	3	50	3	80	3	30

Figure 4B

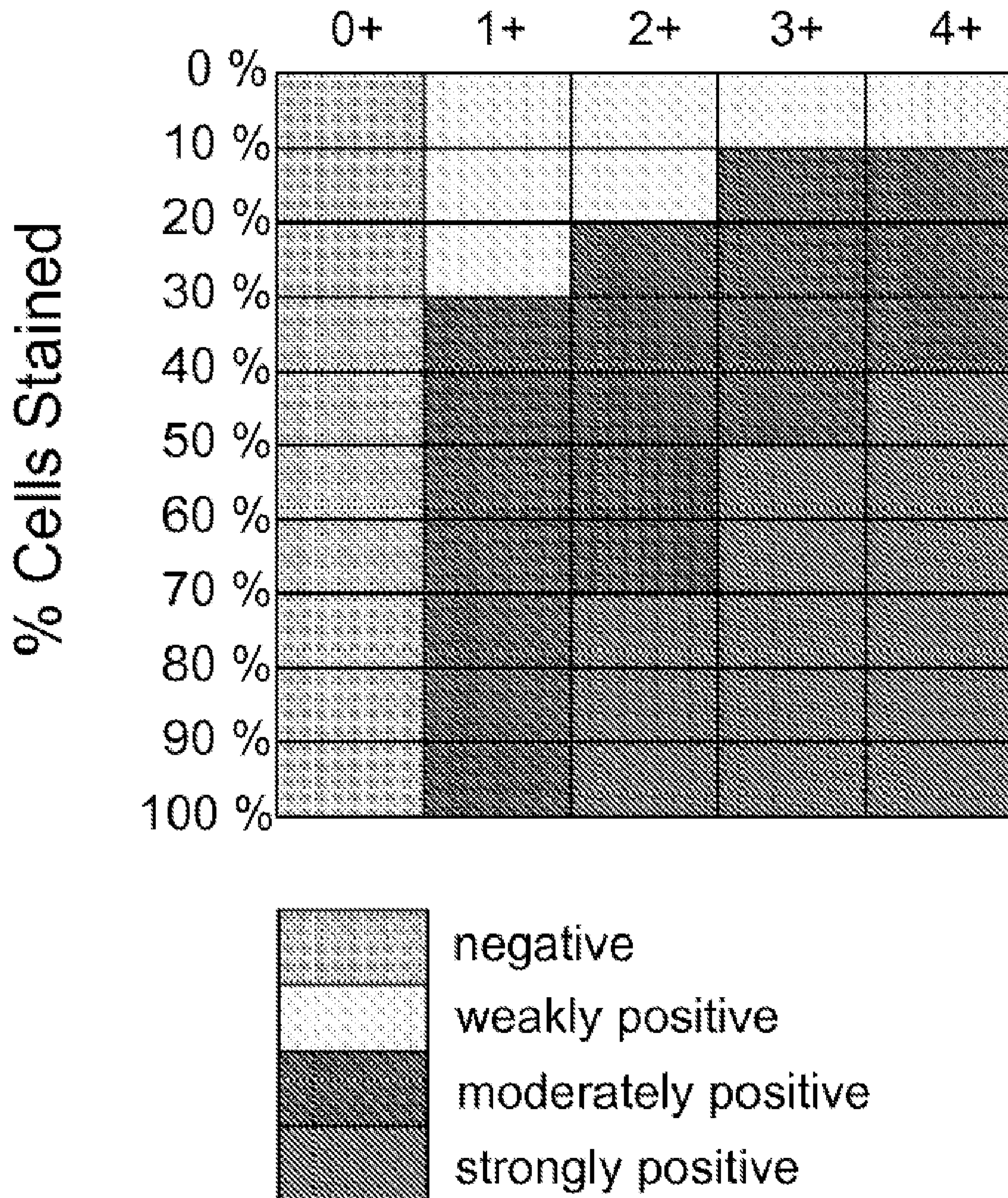


Figure 5

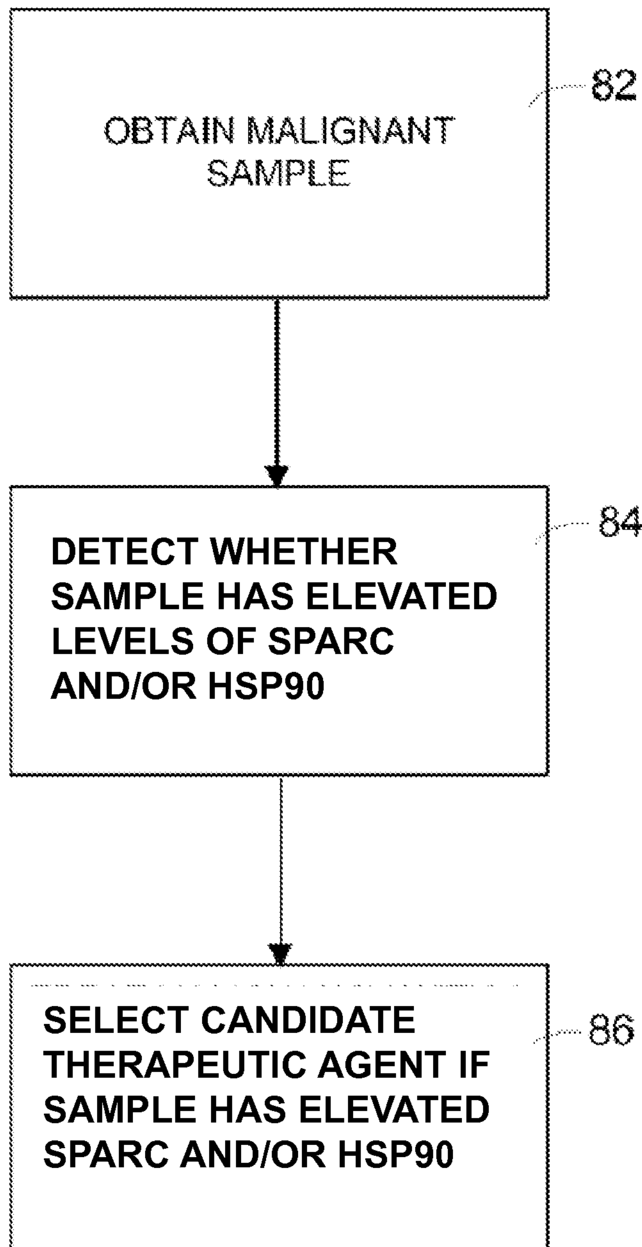


Figure 6

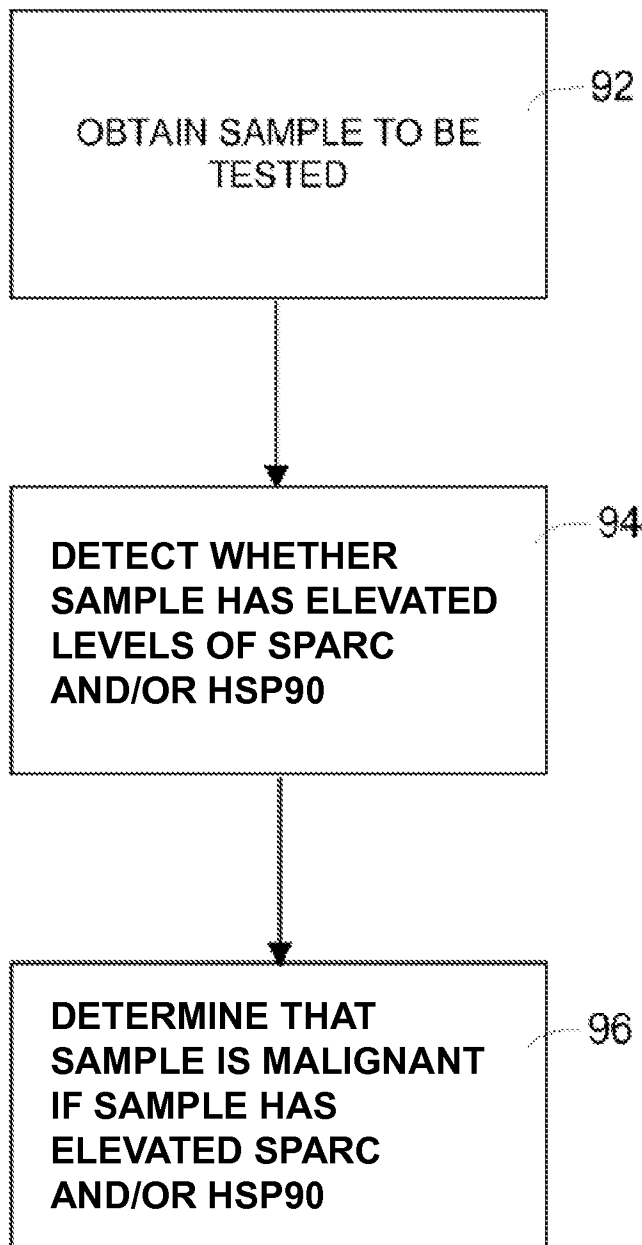


Figure 7

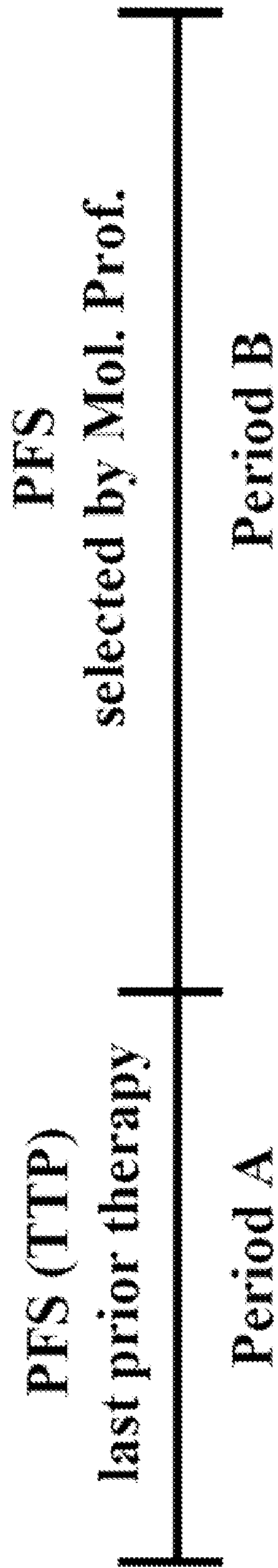


Figure 8

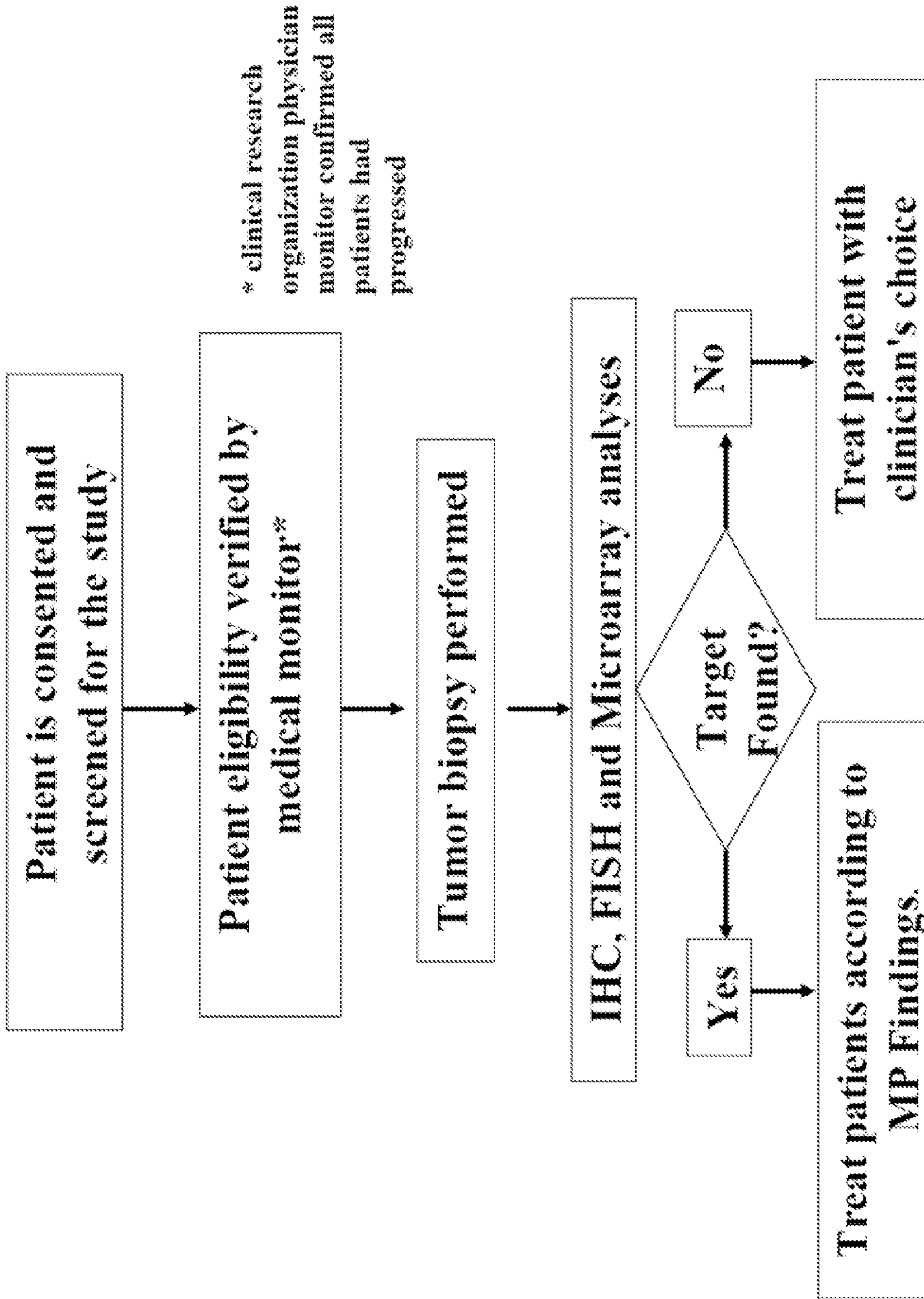


Figure 9

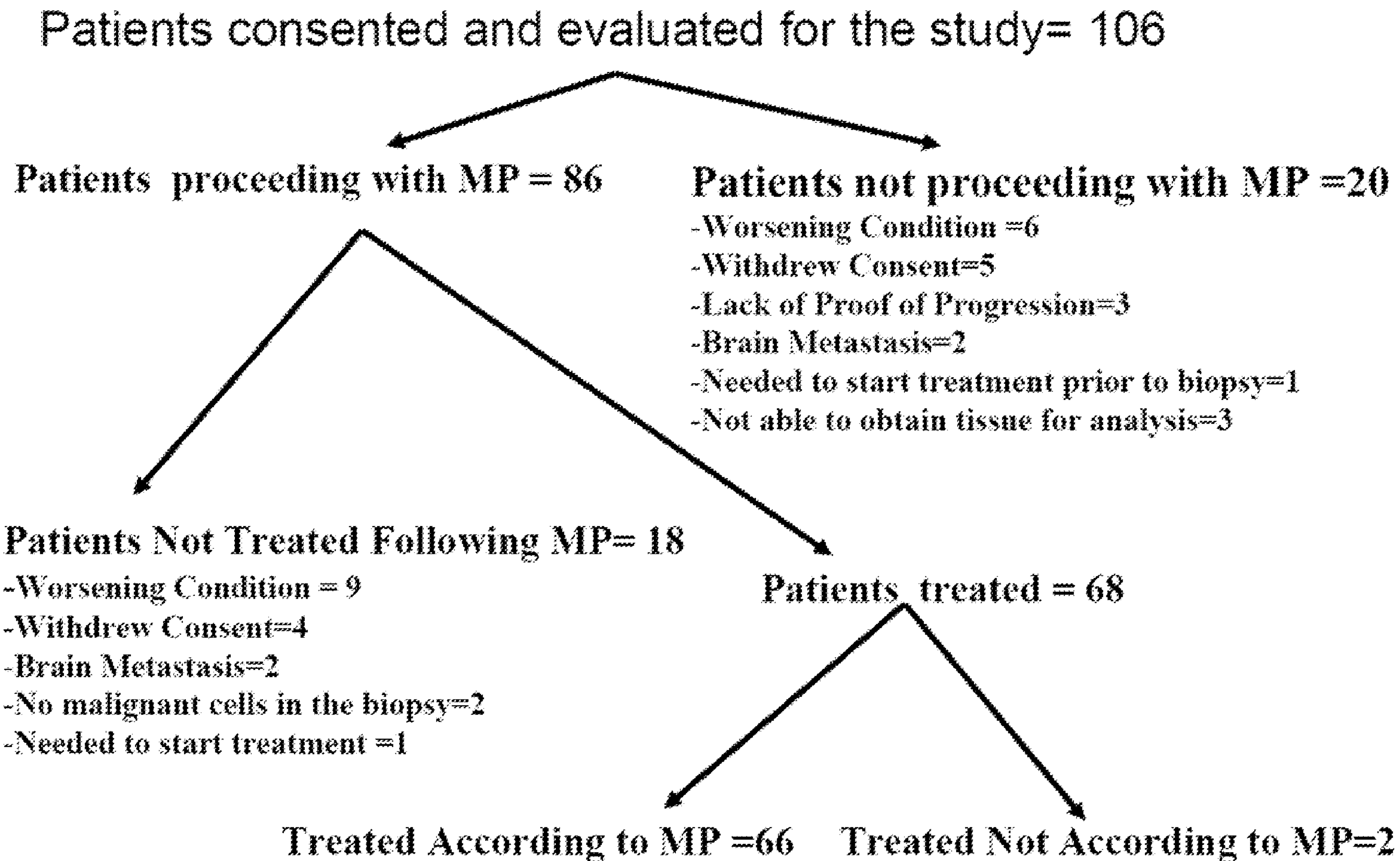


Figure 10

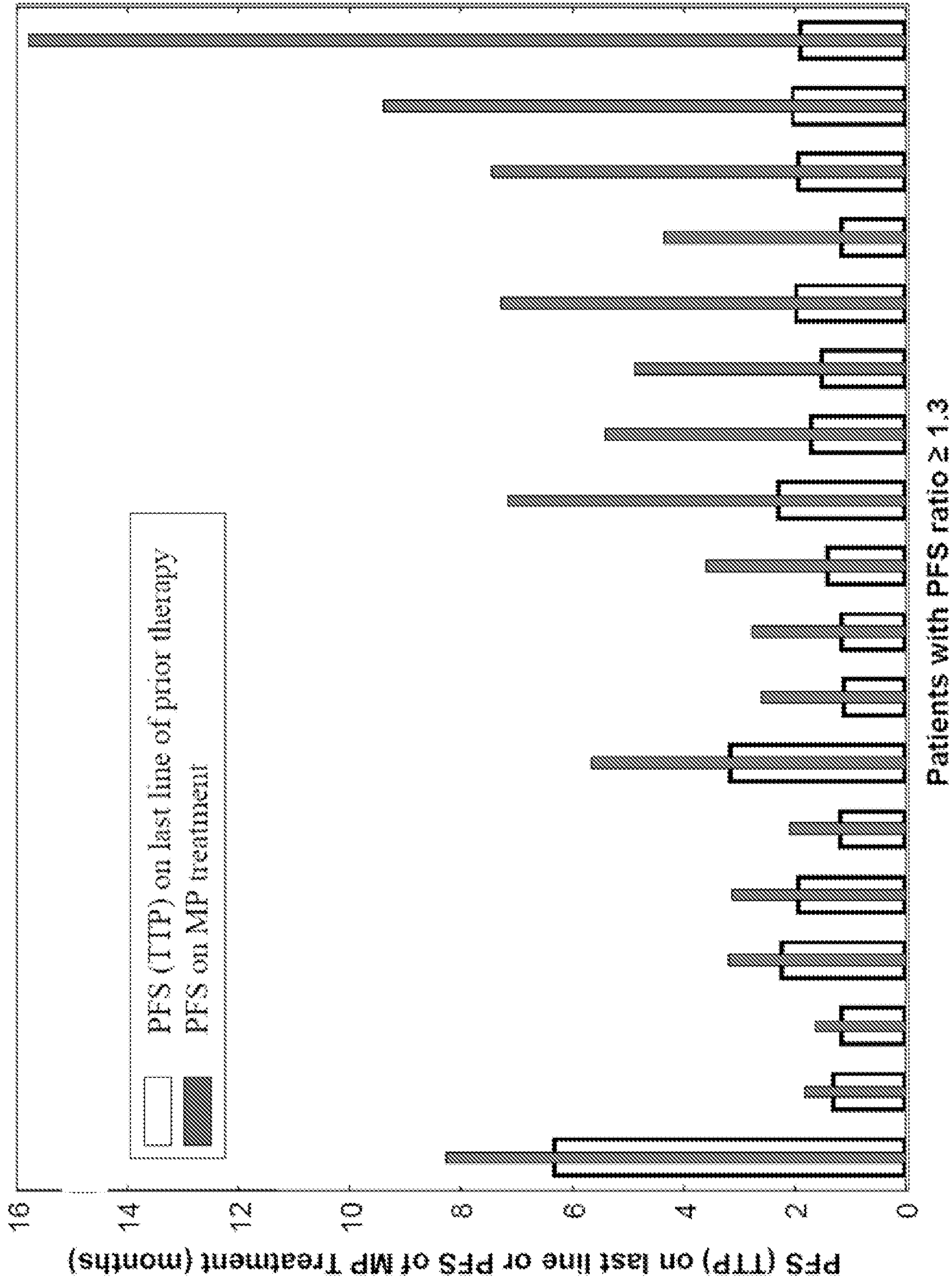


Figure 11

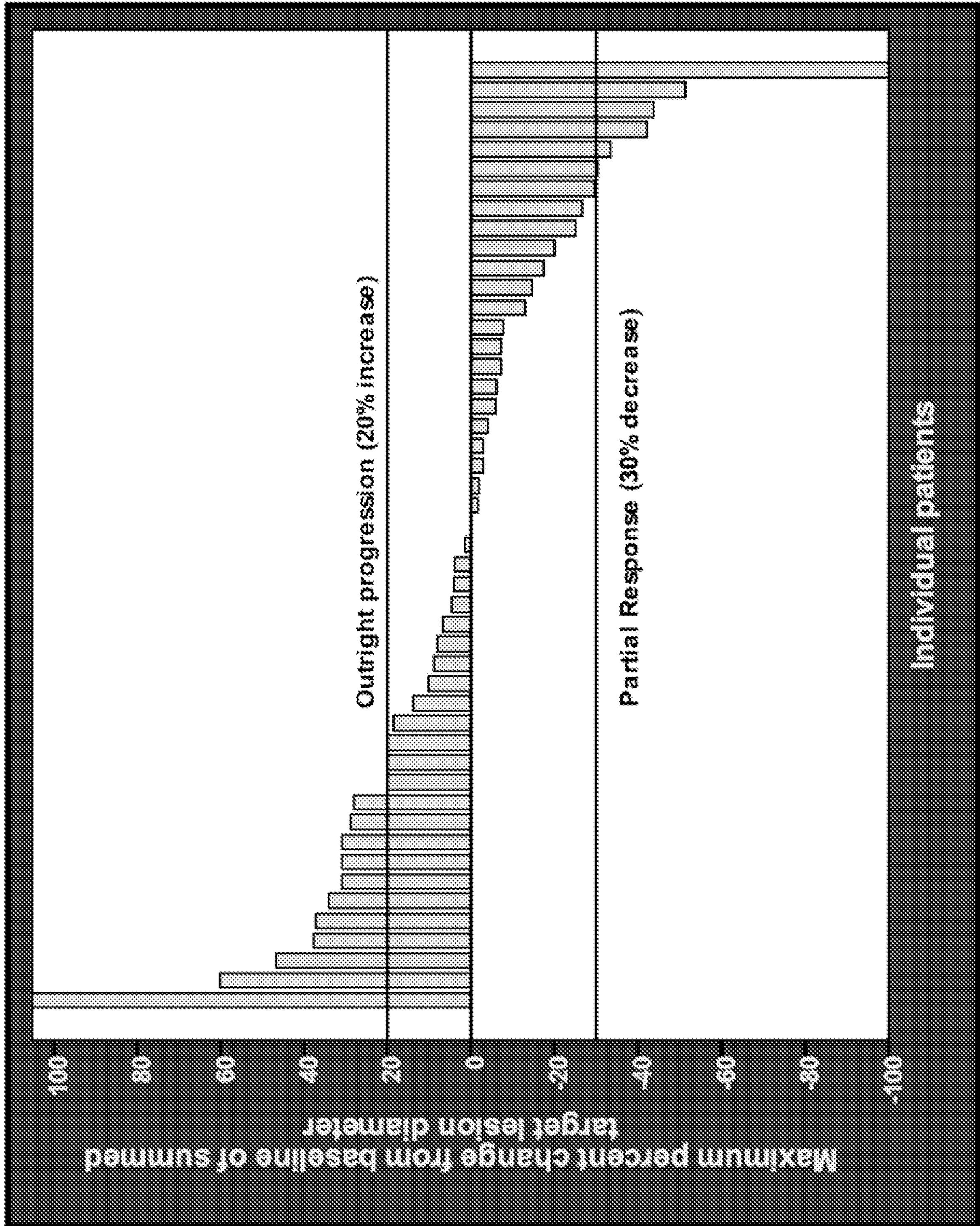


Figure 12

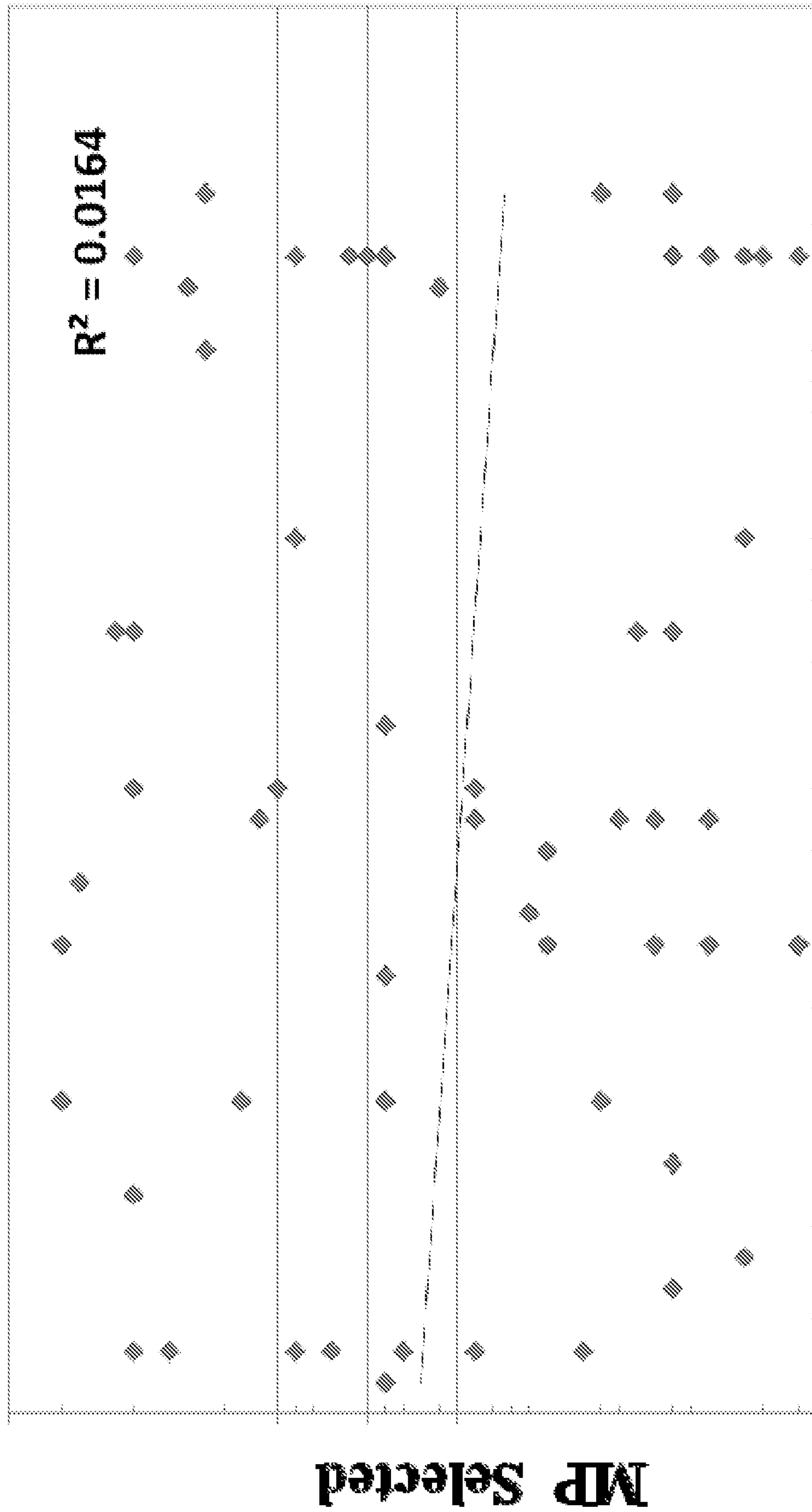


Figure 13

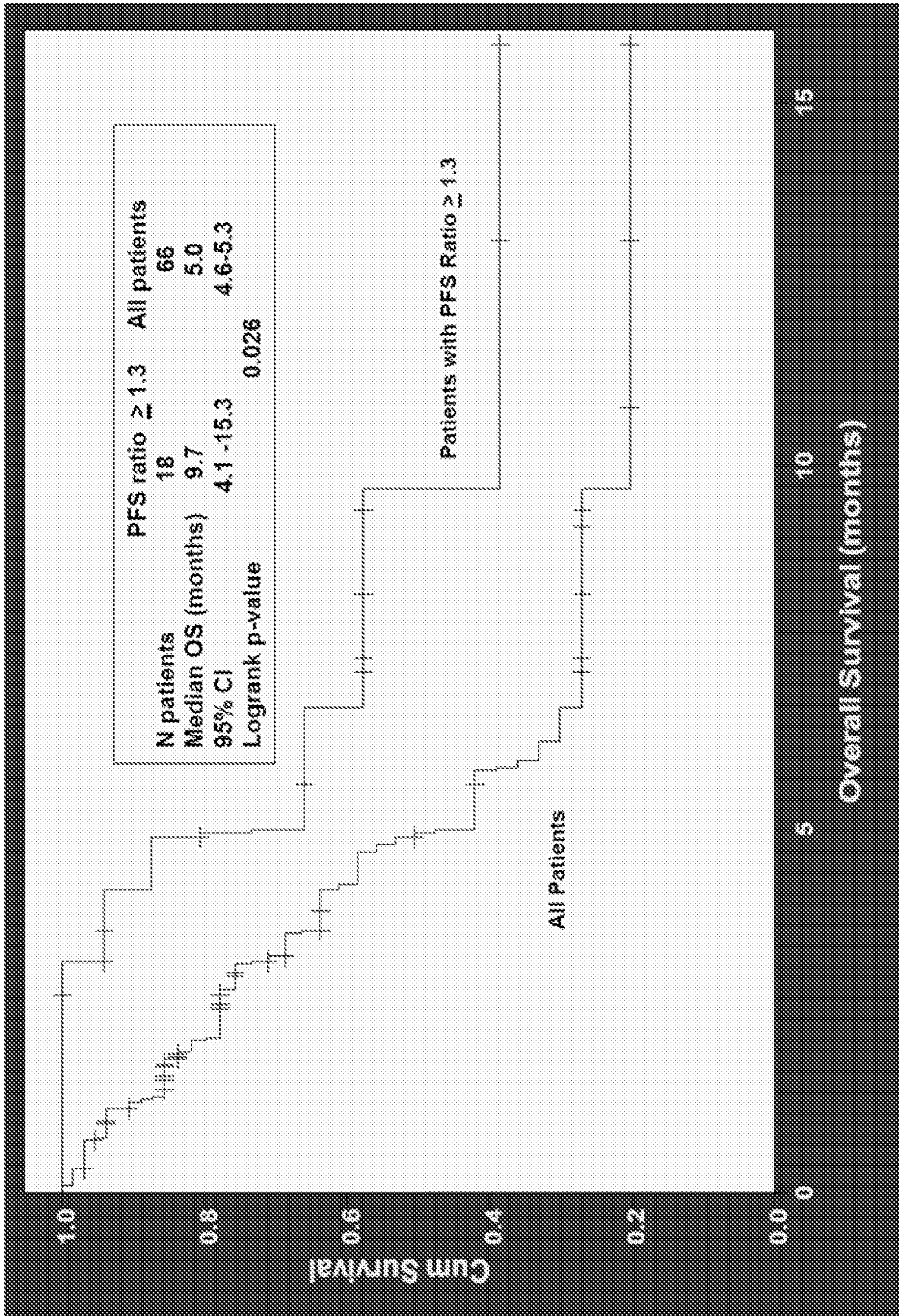


Figure 14

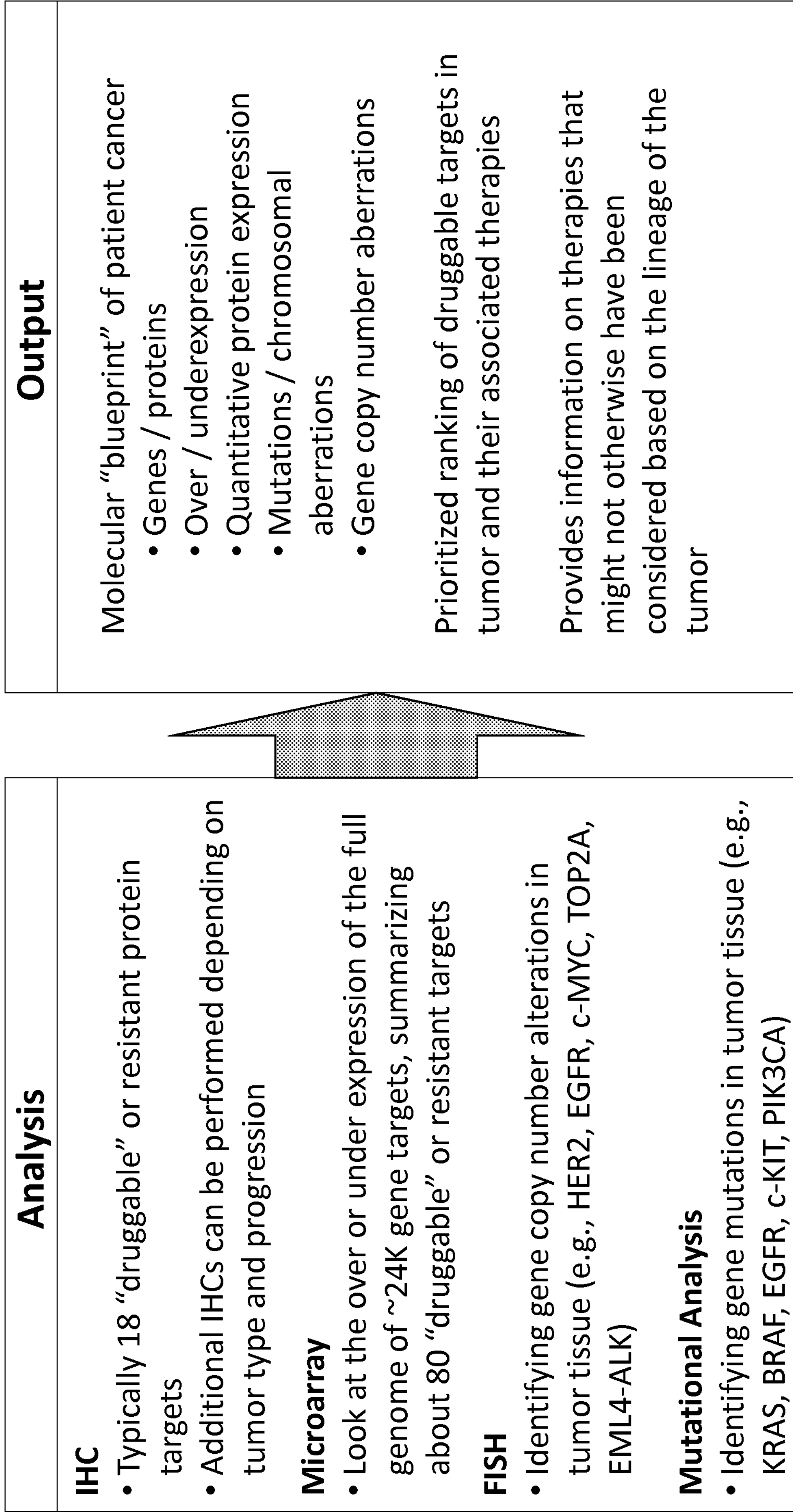


Figure 15

CARIS TARGET NOW
Molecular Profiling Therapeutic Guidance

CARIS LIFE SCIENCES

Patient Information	Specimen Information	Ordered By
TEST, PATIENT **Case Number:** TN10-100367 **Date Of Birth:** 01/01/1900 **Sex:** Male **SSN:** 123-45-6789	**Primary Tumor Site:** Breast **Specimen Site:** Breast **Specimen Collected:** 10/04/2010 **Specimen Received:** 10/05/2010 **Date Reported:**	**TEST TEST** **Test Organization** 123 Test Street Phoenix, AZ 85004 (123) 456-7899

Caris Target Now Summary

Molecular Subtype of Breast Cancer: HER2+

Clinical History

Clinical History goes here

Agents Associated WITH CLINICAL BENEFIT	Agents Associated With LACK OF CLINICAL BENEFIT
Taxanes	**methotrexate**
nab-paclitaxel	**doxorubicin, liposomal-doxorubicin, epirubicin**
5-fluorouracil	**trastuzumab**
lapatinib	**temozolomide**
anastrozole, letrozole	**cytarabine**
tamoxifen, chemotherapy	
irinotecan	
erlotinib, gefitinib	
cetuximab, panitumumab	
hydroxyurea	
gemcitabine	
pentostatin	

Specimens Received

The specimens consist of:

1 (A) Formalin Fixed, site: Breast - Client ID(123456).

The role of Target Now is to identify biomarkers and therapies associated with clinical benefit or lack of clinical benefit for cancer patients. The selection of any, all or none of the matched agents resides with the discretion of the treating physician. **If a patient's tumor has previously progressed on an agent identified as associated with clinical benefit on this report, the patient should not be re-treated with this agent.**

An expert oncology consultation can be arranged if a request is made through our Client Services Department at 1-800-901-5177.

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FIGURE 16A

TARGET NOW® SUMMARY - AGENTS ASSOCIATED WITH CLINICAL BENEFIT

Agents Associated With CLINICAL BENEFIT	Biomarker	Result	Method	Summary Statement
Taxanes	TLE3	Above Threshold	IHC	High expression of TLE3 has been associated with response to taxane therapy.
nab-paclitaxel	SPARC Monoclonal	Above Threshold	IHC	High expression of SPARC has been associated with benefit from nab-Paclitaxel.
5-fluorouracil	TS	Negative	IHC	Low expression of TS has been associated with benefit from fluoropyrimidines.
lapatinib	Her2/Neu	Not Amplified	FISH	Although HER2 is not amplified, HER2 targeted protein kinase inhibitors are of potential benefit due to overexpression of HER2.
	Her2/Neu	Above Threshold	IHC	
anastrozole, letrozole	PR	Above Threshold	IHC	High expression of ER and PR have been associated with benefit from aromatase inhibitor.
	ER	Above Threshold	IHC	
tamoxifen, chemotherapy	PR	Above Threshold	IHC	High expression of ER, PR and Ki67 have been associated with benefit from tamoxifen and chemotherapy.
	ER	Above Threshold	IHC	
	Ki67	Above Threshold	IHC	
irinotecan	TOPO1	Above Threshold	IHC	High expression of TOPO1 has been associated with benefit from irinotecan.
erlotinib, gefitinib	PTEN	Above Threshold	IHC	High expression of PTEN and amplification/ polysomy of EGFR have been associated with benefit from EGFR targeted tyrosine kinase inhibitors.
	EGFR	Positive	FISH	
cetuximab, panitumumab	EGFR	Positive	FISH	High expression of PTEN and amplified/polysomic EGFR have been associated with benefit from EGFR-targeted antibodies.
	PTEN	Above Threshold	IHC	
hydroxyurea	RRM1	Under Expressed	Microarray-Illumina	Low expression of RRM1 has been associated with benefit from hydroxyurea.
gemcitabine	DCK	Over Expressed	Microarray-Illumina	Low expression of RRM1 and high expression of DCK, have been associated with benefit from gemcitabine.
	RRM1	Under Expressed	Microarray-Illumina	
pentostatin	ADA	Over Expressed	Microarray-Illumina	High expression of ADA has been associated with benefit from pentostatin.

Patient: TEST, PATIENT **TN10-100367** **Physician: TEST TEST**

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FIGURE 16B

TARGET NOW® SUMMARY - Agents Associated with LACK OF CLINICAL BENEFIT

Agents Associated With LACK OF CLINICAL BENEFIT	Biomarker	Result	Method	Summary Statement
methotrexate	MRP1	Above Threshold	IHC	High expression of MRP1 and DHFR has been associated with lack of benefit from combination therapies containing methotrexate.
	DHFR	Over Expressed	Microarray-Illumina	
doxorubicin, liposomal-doxorubicin, epirubicin	TOP2A	Negative	IHC	Although TOPO2A is amplified, anthracyclines are potentially of minimal benefit due to low expression of TOPO2A, high expression of PGP and lack of HER2 amplification.
	Her2/Neu	Not Amplified	FISH	
	TOP2A	Amplified	FISH	
	PGP	Above Threshold	IHC	
trastuzumab	PTEN	Above Threshold	IHC	Although PTEN is above threshold, HER2 targeted antibody is potentially of minimal benefit due to lack of HER2 amplification and mutated PIK3CA.
	Her2/Neu	Above Threshold	IHC	
	PIK3CA	Mutated	Molecular	
	Her2/Neu	Not Amplified	FISH	
temozolomide	MGMT	Above Threshold	IHC	High expression of MGMT has been associated with lack of benefit from temozolomide.
cytarabine	CDA	Over Expressed	Microarray-Illumina	High expression of CDA and DCK have been associated with lack of benefit from cytarabine.
	ADA	Over Expressed	Microarray-Illumina	
	DCK	Over Expressed	Microarray-Illumina	

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FIGURE 16C

IHC Biomarker Detail

Biomarker	Significant Result	Patient Tumor		Histoscore	Prognosis	Threshold Biomarker Intensity/Percentage
		Staining Intensity	Percent Staining			
ER	✓	3	70	4.0	**Favorable**	=0+ or >0+ and ≥1%
Her2/Neu	✓	3	55	3.0	**Unfavorable**	<2+ or =2+ and <10% or =3+ and ≥30%
ECAD		2	40	5.0	**Favorable**	=0+ or <2+ and <10% or =2+ and ≥30% or ≥3+
PR	✓	2	40	5.0	**Favorable**	=0+ or >0+ and ≥1%
Ki67	✓	2	30	6.0	**Unfavorable**	=0+ or <15% or ≥1+ and ≥20%
p53		1	55	4.0	**Favorable**	=0+ or ≥1+ and ≥10%

* Staining intensity based on 0 (neg) to 3+ (highest). Percent Positive Average: Percent of tumor cells staining. These tests were performed by IHC in conjunction with automated image analysis on ACIS III(Dako). The performance characteristics of the above test have been determined at Caris Life Sciences. While some antibodies have not been approved by the FDA, clearance/approval is not mandated. These antibodies are well documented and clinically accepted prognostic indicators. These tests should not be regarded as part of research investigations. Known positive and negative control tissue show appropriate staining. Tumor bio-markers results to be used in context with clinical/pathological findings. College of American Pathologists (CAP)-required information for predictive/prognostic markers: Type of specimen fixation and detection system: Polymer detection kits are used on formalin-fixed paraffin-embedded sections. Clones used: ER(1D5), PR(PgR636), Her-2(A0485), p53(DO-7), Ki-67(mib-1).

** All significant results are reflected in the Target Now Summary.

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FIGURE 16D

Biomarker	Significant Result	Patient Tumor		Threshold
		Staining Intensity	Percent Staining	Biomarker Intensity/Percentage
TLE3	✓	3	80	=0+ and =100% or ≥1+ and ≥10%
p95		3	70	=0+ and =100% or ≥2+ and ≥30%
MGMT	✓	3	60	=0+ or =1+ and <10% or ≥1+ and ≥50%
MRP1	✓	3	35	=0+ or =1+ and <10% or ≥1+ and ≥10%
BCRP		2	75	=0+ or =1+ and <10% or ≥1+ and ≥10%
PTEN	✓	2	70	=0+ or =1+ and ≤10% or ≥2+ and ≥10%
c-kit		2	60	=0+ and =100% or ≥2+ and ≥30%
SPARC Monoclonal	✓	2	50	=0+ and =100% or ≥2+ and ≥30%
CK 5/6		2	45	=0+ and =100% or ≥2+ and ≥30%
TOPO1	✓	2	35	=0+ or <10% or ≥1+ and ≥10%
ERCC1		2	30	=0+ or =1+ and ≤25% or ≥2+ and ≥50%
CK17		1	60	=0+ and =100% or ≥2+ and ≥30%
RRM1		1	60	=0+ or ≥2+ and ≥50%
PGP	✓	1	50	=0+ or =1+ and <10% or ≥1+ and ≥10%
SPARC Polyclonal		1	45	=0+ and =100% or ≥2+ and ≥30%
CAV-1		1	25	=0+ and =100% or ≥2+ and ≥30%
CK14		1	20	=0+ and =100% or ≥2+ and ≥30%
Androgen Receptor		0	100	=0+ and =100% or ≥1+ and ≥10%
PDGFR		0	100	=0+ and =100% or ≥2+ and ≥30%
TOP2A	✓	0	100	=0+ or <10% or ≥1+ and ≥10%
TS	✓	0	100	=0+ or =1+ and ≤25% or ≥2+ and ≥30%

* Caris Life Sciences has defined threshold levels of reactivity of IHC to establish cutoff points based on published evidence.
** All significant results are reflected in the Target Now Summary.

Not Signed

Pathologist Comment Regarding Results Above
Since PTEN immunoreactivity by IHC was not absent, additional testing for EGFR (by FISH) was performed and (depending on tumor type) for EGFR mutational analysis, KRAS and BRAF as well (see corresponding results in this report).

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FIGURE 16E

Microarray Analysis of RNA Expression on Formalin Fixed Tissue

Gene	Ratio	Expression*	[illegible]
EPHA2	0.23	Under Expressed	
HIF1A	0.24	Under Expressed	
ABCC1	0.41	Under Expressed	
KIT	0.43	Under Expressed	
PDGFC	0.47	Under Expressed	
YES1	0.48	Under Expressed	
SPARC	0.52	Under Expressed	
RRM1	0.62	Under Expressed	✓
ECGF1	0.64	No Change	
FYN	0.70	No Change	
PTGS2	0.73	No Change	
RRM2	0.75	No Change	
MSH2	0.76	No Change	
PDGFRB	0.79	No Change	
PDGFRA	0.80	No Change	
RXRB	0.80	No Change	
FOLR2	0.83	No Change	

Gene	Ratio	Expression*	[illegible]
MLH1	0.86	No Change	
TOP2A	0.92	No Change	
VEGFA	0.94	No Change	
PTEN	0.97	No Change	
TXNRD1	1.02	No Change	
ERCC3	1.05	No Change	
RAF1	1.07	No Change	
MGMT	1.08	No Change	
HDAC1	1.14	No Change	
HSP90AA1	1.17	No Change	
CES2	1.17	No Change	
SSTR2	1.21	No Change	
DNMT1	1.21	No Change	
GART	1.24	No Change	
GSTP1	1.24	No Change	
KDR	1.27	No Change	
TOP2B	1.27	No Change	

Gene	Ratio	Expression*	[illegible]
POLA1	1.55	No Change	
ASNS	1.68	No Change	
GNRH1	1.70	No Change	
TYMS	1.81	No Change	
TK1	1.89	Over Expressed	
BIRC5	1.93	No Change	
ADA	1.95	Over Expressed	✓
NFKB1	2.50	Over Expressed	
DHFR	2.71	Over Expressed	✓
LYN	2.76	Over Expressed	
RRM2B	2.92	Over Expressed	
OGFR	3.48	Over Expressed	
CDA	3.90	Over Expressed	✓
DCK	4.21	Over Expressed	✓
CD33	5.51	Over Expressed	
VHL	5.55	Over Expressed	
HCK	5.94	Over Expressed	

* "No Change" indicates that there is no difference in expression for this gene between the tumor and control tissues at a significance level of p<=0.001. A significance level of p<=0.001 has been chosen since genes passing this threshold can be validated as differentially expressed by alternative methods approximately 95% of the time.

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FIGURE 16F

Not Informative*

Genes						
ABCG2	CD52	ERBB2	IL2RA	PGR	SSTR1	TNF
AR	DNMT3A	ERCC1	LCK	RARA	SSTR3	TOP1
BCL2	DNMT3B	ESR1	MS4A1	RXRG	SSTR4	VDR
BRCA1	EGFR	FLT1	NFKB2	SRC	SSTR5	ZAP70
BRCA2						

* "Not Informative" indicates that the data obtained for either the patient sample or the control sample were not of sufficiently high quality to confidently evaluate the expression level of that particular RNA transcript.

Methodology

Total RNA is extracted from tumor tissue and is converted to cDNA. This cDNA sample is then subjected to a whole genome (24K) microarray analysis using Illumina cDNA-mediated annealing, selection, extension and ligation (DASL) process. The expression of a subset of 80 genes are then compared to a tissue specific normal control and the relative expression ratios of these 80 target genes is determined as well as the statistical significance of the differential expression.

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FIGURE 16G

ANALYSIS BY FISH

Gene / TSCN	Cells Counted	Result	Total/Avg Gene Copy Number	% of Cells	Total/Avg Control Copy Number	Ratio Calculation	Ratio	[illegible]
EGFR nuc ish (D7Z1x,EGFRx)[/100]	100	Positive	3.54	40.00%	1.23	EGFR / CEP 7	2.88	✓
	Reference Range: Positivity for increased gene copy number by FISH has been defined as ≥ 4 copies in 40% or more tumor cells. Gene amplification is defined by the presence of a gene/ chromosome per cell ratio of ≥ 2, or ≥ 15 copies of the genes per cell in ≥ 10% of analyzed cells.							
Her2/Neu nuc ish (D17Z1x1-2,HER2x1-2)[/30]	30	Not Amplified	2.75	30.00%	2.42	HER-2/neu / Chromosome 17	1.14	✓
	Reference Range: HER2/neu:CEP 17 signal ratio of ≥2.0; and non-amplification as <2.0 per Abbott (Pathvysion) and Herceptin package inserts. Per ASCO CAP guidelines, FISH amplification is > 2.2 and non-amplification is < 1.8. Please note, the range 1.8-2.2 is equivocal. Equivocal breast cancers falling in the 2.0-2.2 range are regarded as tumors with low level amplification and are eligible for consideration for Herceptin Therapy.							
TOP2A nuc ish(D17Z1x1-2,TOP2Ax1-2)[/100]	100	Amplified	4.52	30.00%	1.23	TOP2 / CEP 17	3.67	✓
	Reference Range: In breast cancer, FISH amplification has been established as a TOP2:CEP 17 signal ratio of ≥2.0. A positive value suggests a shorter recurrence-free survival, and overall survival. Nielsen KV, et al., The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCC trial 89D. Acta Oncologica 47:725-734, 2008.							
cMYC nuc ish (D8Z2x1-2,cMYCx1-2)[/100]	100	Amplified	2.56	30.00%	1.13	cMYC / CEP8	2.27	
	Reference Range: In breast cancer, FISH amplification has been established as a cMYC:CEP8 signal ratio of ≥2.0. A positive value suggests a poor prognosis, early recurrence, and shorter disease survival.							

These tests were developed and their performance characteristics determined by Caris Life Sciences, Inc. The HER2 probe has been cleared by the US Food and Drug Administration. Probes for TOP2, cMYC and EGFR have not been cleared or approved by the US Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. These tests are used for clinical purposes. They should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high complexity clinical testing.

Not Signed

Comments on Analysis By FISH

Fluorescence in situ hybridization (FISH) was performed with a probe specific for HER-2/neu and a probe for the pericentromeric region of chromosome 17 (Vysis). Interphase nuclei were examined by two observers and the ratio of HER2/neu signals to chromosome 17 signals was 1.136 to 1, indicating NO AMPLIFICATION of this gene.
Fluorescence in situ hybridization (FISH) was carried out using a probe specific for cMYC and a probe for the pericentromeric region of chromosome 8 (Vysis). Interphase nuclei were examined by two observers and the ratio of cMYC signals to chromosome 8 centromeric signals was 2.265 to 1, indicating AMPLIFICATION of this gene.
The patient's tissue revealed the presence of an average of >4.00 copies/cell of the EGFR gene located at 7p12 in 50% of cells examined. Within the limits of the technology, this sample is considered POSITIVE for EGFR. In addition, amplification of the EGFR gene WAS observed. These results should be used with caution, and correlation with clinicopathologic findings is recommended.
Fluorescence in situ hybridization (FISH) was carried out using a probe specific for TOP2 and a probe for the pericentromeric region of chromosome 17 (Vysis). Interphase nuclei were examined by two observers and the ratio of TOP2 signals to chromosome 17 signals was 3.675 to 1, indicating AMPLIFICATION of this gene.

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FIGURE 16H

MUTATIONAL ANALYSIS

Gene	Analysis	Result	Significant Result
PIK3CA	Positive for a mutation in exon (9) of the PIK3CA.	Mutated	✓
	Procedure: Direct sequence analysis was performed on genomic DNA isolated from a formalin-fixed paraffin-embedded tumor sample using custom nested M13 linked primers designed to flank, amplify, and sequence selected regions of exons 9 (codons 539-546) and 20 (codons 1043-1049) of the PIK3CA gene located at 3q26.3. This test has a sensitivity to detect as low as approximately 20% population of cells containing a mutation in exons 9 or 20 in a background of non-mutant or normal (wild type) cells.		

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Comments on Molecular Profile Analysis

PIK3CA Sequencing mutation present.

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FIGURE 16I

Appendix

BIOMARKER DESCRIPTION

Target	Biomarker Description
EGFR	EGFR (epidermal growth factor receptor) is a receptor tyrosine kinase and its abnormalities contribute to the growth and proliferation of many human cancers. EGFR inhibitors such as erlotinib and gefitinib as well as the anti-EGFR antibodies cetuximab and panitumumab are available for targeting the EGFR pathway. EGFR has been linked to response to EGFR targeted therapies.
ER	The estrogen receptor (ER) is a member of the nuclear hormone family of intracellular receptors which is activated by the hormone estrogen. Its main function is as a DNA binding transcription factor to regulate estrogen mediated gene expression. ER is expressed in breast, ovarian and endometrial tissue. Estrogen and its receptors are essential for sexual development and reproductive function, but also play a role in other tissues such as bone. Estrogen receptors are over-expressed in many breast cancer cases, referred to as "ER positive." Estrogen binding to ER on cancer cells leads to cancer cell proliferation. Breast tumors marked by ER positivity currently form the basis of selecting patients who will receive and benefit from hormone based therapy.
Her2/Neu	ErbB2/Her2 encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. Her2 has no ligand binding domain of its own and therefore cannot bind growth factors. It does however bind tightly to other ligand-bound EGF receptor family members to form a heterodimer and enhances kinase-mediated activation of downstream signaling pathways leading to cell proliferation. Her2 is overexpressed in 15-30% of newly diagnosed breast cancers. Clinically, Her2 is a target for the monoclonal antibody Trastuzumab/Herceptin (which binds and blocks the receptor extracellularly) and the kinase inhibitor Lapatinib/Tykerb (which binds and blocks the receptor intracellularly).
Ki67	Ki67 is a nuclear antigen expressed during all active phases of the cell cycle, but is absent from resting cells. It is a well-established marker of cell proliferation and its overexpression has been associated with poor prognosis and aggressive disease. Presence of Ki67 in hormone-receptor positive breast cancer is associated with better response to chemoendocrine therapy.
MGMT	O-6-methylguanine-DNA methyltransferase (MGMT) encodes a DNA repair enzyme. Loss of MGMT expression leads to compromised DNA repair in cells and may play a significant role in cancer formation. Low MGMT expression has been correlated with response to temozolomide.
MRP1	MRP1 (multidrug resistance–associated protein 1) is one of several drug resistance proteins identified to date and is an important mediator of the Multi Drug Resistance (MDR) phenotype in cancer cells. MRP1 is found to confer a lack of response to anthracyclines (eg daunorubicin, doxorubicin), vinca alkaloids (vincristine, vinblastine), epipodophylotoxins (etoposide and teniposide), and mitoxantrone, but probably not taxanes (Paclitaxel, Docetaxel), by causing the efflux of glutathione-conjugated natural product agents (glutathione is a peptide composed of 3 amino-acids). Elevated levels of MRP1 have been observed in relapsed acute myelogenous leukemia, chronic lymphatic leukemia, small-cell and non–small-cell lung cancer, and neuroblastoma among other malignancies.
PGP	P-glycoprotein (MDR1, ABCB1) is an ATP-dependent, transmembrane drug efflux pump with broad substrate specificity, which pumps antitumor drugs out of cells. Its expression is often induced by chemotherapy drugs and is thought to be a major mechanism of chemotherapy resistance. Overexpression of p-gp can be a negative predictive factor for various drugs such as anthracylines (doxorubicin, epirubicin), paclitaxel, vinblastine etc. P-gp remains the most important and dominant representative of Multi Drug Resistance phenotype and is correlated with disease state and resistant phenotype.
PIK3CA	The hot spot missense mutations in the gene encoding the p110alpha subunit of PI3 kinase, PIK3CA are present in 25-30% of human breast cancers, resulting in activation of the PI3 kinase pathway. Patients with activated PI3K pathway due to either PTEN loss or PIK3CA mutation have a significantly shorter survival following trastuzumab treatment.
PR	The progesterone receptor (PR or PGR) is an intracellular steroid receptor that specifically binds progesterone, an important hormone that fuels breast cancer growth. Routinely, breast tumors are analyzed for PR positivity along with ER (estrogen receptor). PR positivity in the tumor indicates that the tumor is more likely to be responsive to hormone therapy by drugs such as tamoxifen
PTEN	PTEN (phosphatase and tensin homolog) is a tumor suppressor gene that prevents cells from growing and dividing too rapidly and out of control. PTEN removes phosphate groups from a molecule called PIP3 to generate PIP2. This dephosphorylation helps to ensure the inhibition of the AKT cell survival pathway. PTEN protein is found in all types of cells and loss of PTEN function is one of the most common occurrences in multiple advanced human cancers. PTEN is an important mediator in signaling downstream of EGFR, which can be blocked by EGFR and Her2 targeted therapies. Loss of PTEN function is associated with a lack of response to these therapies.
SPARC Monoclonal	SPARC Monoclonal (secreted protein acidic and rich in cysteine) is a calcium-binding matricellular glycoprotein secreted by many types of cells. It has a normal role in wound repair, cell migration, and cell-matrix interactions. Its over-expression is thought to have a role in tumor invasion and angiogenesis. A few studies indicate that SPARC over-expression improves the response to the anti cancer drug, nab-paclitaxel. The improved response is thought to be related to SPARC's role in accumulating albumin and albumin targeted agents within tumor tissue.
TLE3	TLE3 is a member of the transducin-like enhancer of split (TLE) family of proteins that have been implicated in the tumorigenesis and classification of sarcomas. TLE3 is a nuclear expressed protein originally identified in Drosophila as required for epithelial cell fate determination through interacting with members of the notch and wnt pathway. It acts downstream of APC and β-catenin to repress transcription of a number of oncogenes, which influence growth and microtubule stability. Recent studies indicate that TLE3 expression is associated with response to taxane therapy in triple negative breast cancer patients.
TOP2A	TOPOIIA is an enzyme that alters the super coiling of double stranded DNA and allows chromosomal segregation into daughter cells. Due to its essential role in DNA synthesis and repair, and frequent over expression in tumors, TOPOIIA is an ideal target for antineoplastic agents. High expression and/or co-amplification of TOPOIIA and HER2 have been associated with benefit from anthracycline based therapy.

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FIGURE 16J

BIOMARKER DESCRIPTION

Target	Biomarker Description
TOPO1	Topoisomerase I is an enzyme that alters the supercoiling of double-stranded DNA. TopoI acts by transiently cutting one strand of the DNA to relax the coil and extend the DNA molecule. The regulation of DNA supercoiling is essential to DNA transcription and replication, when the DNA helix must unwind to permit the proper function of the enzymatic machinery involved in these processes. Higher expression of TopoI has been associated with response to first line chemotherapy containing Irinotecan, a TopoI inhibitor.
TS	Thymidylate synthetase (TS) is an enzyme that generates thymidine monophosphate (dTMP), which get phosphorylated to thymidine triphosphate (dTTP) for use in DNA synthesis and repair. The reactions catalyzed by TS also yield dihydrofolate as a secondary product. As an anti-cancer chemotherapy target, thymidylate synthetase can be inhibited by fluoropyrimidine or certain folate analogues. High TS has been associated with lack of response to fluoropyrimidine whereas low or no TS expression has been associated with improved clinical response to fluoropyrimidine.

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FIGURE 16K

LITERATURE LEVEL of EVIDENCE

Agents Associated With CLINICAL BENEFIT	Reference	Level of Evidence
5-fluorouracil	Yu, Z., J. Sun, et al. (2005). "Thymidylate synthase predicts for clinical outcome in invasive breast cancer." Histol Histopathol 20(3): 871-8.	**II-3 / Good**
5-fluorouracil	Toi, M., T. Ikeda, et al. (2007). "Predictive implications of nucleoside metabolizing enzymes in premenopausal women with node-positive primary breast cancer who were randomly assigned to receive tamoxifen alone or tamoxifen plus tegafur-uracil as adjuvant therapy." Int J Oncol 31(4): 899-906.	**II-3 / Good**
anastrozole, letrozole	Viale, G., M. M. Regan, et al. (2008). "Chemoendocrine compared with endocrine adjuvant therapies for node-negative breast cancer: predictive value of centrally reviewed expression of estrogen and progesterone receptors--International Breast Cancer Study Group." J Clin Oncol 26(9): 1404-10.	**II-3 / Good**
anastrozole, letrozole	Yamashita, H., Y. Yando, et al. (2006). "Immunohistochemical evaluation of hormone receptor status for predicting response to endocrine therapy in metastatic breast cancer." Breast Cancer 13(1): 74-83.	**II-3 / Good**
anastrozole, letrozole	Stendahl, M., L. Ryden, et al. (2006). "High progesterone receptor expression correlates to the effect of adjuvant tamoxifen in premenopausal breast cancer patients." Clin Cancer Res 12(15): 4614-8.	**I / Good**
cetuximab, panitumumab	De Roock, W., S. Tejpar, et al. (2008). "KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab." Ann Oncol 19(3): 508-15.	**II-3 / Fair**
cetuximab, panitumumab	Cappuzzo, F.,P.A. Janne, et al. (2008). "Primary resistance to cetuximab therapy in EGFR FISH-positive colorectal cancer patients." Br J Cancer 99(1): 83-9.	**II-2 / Good**
cetuximab, panitumumab	Personeni, N., S. Tejpar, et al. (2008). "Clinical usefulness of EGFR gene copy number as a predictive marker in colorectal cancer patients treated with cetuximab: a fluorescent in situ hybridization study." Clin Cancer Res 14(18): 5869-76.	**II-3 / Fair**
erlotinib, gefitinib	Cappuzzo, F., F. R. Hirsch, et al. (2005). "Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer." J Natl Cancer Inst 97(9): 643-55.	**II-3 / Good**
erlotinib, gefitinib	Agulnik, M., G. da Cunha Santos, et al. (2007). "Predictive and pharmacodynamic biomarker studies in tumor and skin tissue samples of patients with recurrent or metastatic squamous cell carcinoma of the head and neck treated with erlotinib." J Clin Oncol 25(16): 2184-90.	**II-3 / Fair**
erlotinib, gefitinib	Mellinghoff, I.K., P.S. Mischel, et al. (2005). "Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors." N Engl J Med 353(19): 2012-24.	**II-3 / Good**
erlotinib, gefitinib	Massarelli, E., I.I. Wistuba, et al. (2007). " KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer." Clin Cancer Res 13(10): 2890-6.	**II-3 / Good**
irinotecan	Naniwa, J., N. Terakawa, et al. (2007). "Genetic diagnosis for chemosensitivity with drug-resistance genes in epithelial ovarian cancer." Int J Gynecol Cancer 17(1): 76-82.	**II-3 / Fair**
irinotecan	Kigawa, J., N. Terakawa, et al. (1999). "Topoisomerase-I activity and response to second-line chemotherapy consisting of camptothecin-11 and cisplatin in patients with ovarian cancer." Int J Cancer 84(5): 521-4.	**II-3 / Fair**
irinotecan	Braun, M.S., M.T. Seymour, et al. (2008). "Predictive biomarkers of chemotherapy efficacy in colorectal cancer: results from the UK MRC FOCUS trial." J Clin Oncol 26(16): 2690-8.	**II-1 / Good**
lapatinib	Xia, W., I. Husain, et al. (2007). "Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers." Cancer Res 67(3): 1170-5.	**II-3 / Good**
lapatinib	Press, M. F., R. S. Finn, et al. (2008). "HER-2 gene amplification, HER-2 and epidermal growth factor receptor mRNA and protein expression, and lapatinib efficacy in women with metastatic breast cancer." Clin Cancer Res 14(23): 7861-70.	**II-1 / Good**
nab-paclitaxel	Raefsky, E., et al. Phase II study of neoadjuvant bevacizumab and trastuzumab administered with albumin-bound paclitaxel (nab paclitaxel) and carboplatin in HER2+ locally advanced breast cancer. J Clin Oncol (May 20 suppl; abstr 627), 2008.	**III / Good**
nab-paclitaxel	Yardley, D.A., et al. Phase II study of neoadjuvant gemcitabine, epirubicin, and albumin-bound nab paclitaxel (GEA) in locally advanced breast cancer with SPARC tumor assessments. J Clin Oncol (May 20 suppl; abstr 603), 2008. 26.	**III / Good**
tamoxifen, chemotherapy	Cheang, M. C., S. K. Chia, et al. (2009). "Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer." J Natl Cancer Inst 101(10): 736-50.	**II-2 / Good**
tamoxifen, chemotherapy	Yamashita, H., Y. Yando, et al. (2006). "Immunohistochemical evaluation of hormone receptor status for predicting response to endocrine therapy in metastatic breast cancer." Breast Cancer 13(1): 74-83.	**II-3 / Good**
tamoxifen, chemotherapy	Hugh, J., J. Hanson, et al. (2009). "Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 trial." J Clin Oncol 27(8): 1168-76.	**II-2 / Good**
Taxanes	Kulkarni, S., D. Hicks, et al. (2009)."TLE3 as a candidate biomarker of response to taxane therapy." Breast Cancer Research 11(2):R17	**II-2 / Good**

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FIGURE 16L

LITERATURE LEVEL of EVIDENCE

Agents Associated With LACK OF CLINICAL BENEFIT	Reference	Level of Evidence
doxorubicin, liposomal-doxorubicin, epirubicin	Durbecq, V., M. Paesmans, et al. (2004). "Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel." Mol Cancer Ther 3(10): 1207-14.	**II-2 / Good**
doxorubicin, liposomal-doxorubicin, epirubicin	Chintamani, J. P. Singh, et al. (2005). "Role of p-glycoprotein expression in predicting response to neoadjuvant chemotherapy in breast cancer--a prospective clinical study." World J Surg Oncol 3: 61.	**II-3 / Good**
doxorubicin, liposomal-doxorubicin, epirubicin	Arriola, E., J. S. Reis-Filho, et al. (2007). "Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer." Br Cancer Res Treat 106: 181-189.	**II-2 / Good**
doxorubicin, liposomal-doxorubicin, epirubicin	Brase, J.C., M. C. Gehrmann, et al. (2010). "ERBB2 and TOP2A in Breast Cancer: A comprehensive analysis of gene amplification, RNA levels, and protein expression and their influence on prognosis and prediction." Clin Cancer Res 16(8): 2391-2401.	**II-3 / Good**
methotrexate	Rudas, M., M. Filipits, et al. (2003). "Expression of MRP1, LRP and Pgp in breast carcinoma patients treated with preoperative chemotherapy." Breast Cancer Res Treat 81(2): 149-57.	**II-3 / Good**
methotrexate	Filipits, M., G. Pohl, et al. (2005). "Clinical role of multidrug resistance protein 1 expression in chemotherapy resistance in early-stage breast cancer: the Austrian Breast and Colorectal Cancer Study Group." J Clin Oncol 23(6): 1161-8.	**II-3 / Fair**
temozolomide	Levin, N., I. Lavon, et al. (2006). "Progressive low-grade oligodendrogliomas: response to temozolomide and correlation between genetic profile and O6-methylguanine DNA methyltransferase protein expression." Cancer 106(8): 1759-65.	**II-3 / Good**
temozolomide	Chinot, O. L., M. Barrie, et al. (2007). "Correlation between O6-methylguanine-DNA methyltransferase and survival in inoperable newly diagnosed glioblastoma patients treated with neoadjuvant temozolomide." J Clin Oncol 25(12): 1470-5.	**II-3 / Good**
temozolomide	Kovacs, K., B. W. Scheithauer, et al. (2008). "MGMT immunoexpression predicts responsiveness of pituitary tumors to temozolomide therapy." Acta Neuropathol 115(2): 261-2.	**III / Fair**
trastuzumab	Fujita, T., H. Doihara, et al. (2006). "PTEN activity could be a predictive marker of trastuzumab efficacy in the treatment of ErbB2-overexpressing breast cancer." Br J Cancer 94(2): 247-52.	**II-3 / Good**
trastuzumab	Berns, K., H. M. Horlings, et al. (2007). "A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer." Cancer Cell 12(4): 395-402	**II-2 / Good**
trastuzumab	Nagata, Y., K. H. Lan, et al. (2004). "PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients." Cancer Cell 6(2): 117-27	**II-3 / Good**
trastuzumab	Toi, M., S. Takashima, et al. (2009). "Lapatinib monotherapy in patients with relapsed, advanced, or metastatic breast cancer: efficacy, safety, and biomarker results from Japanese patients phase II studies." British J of Cancer 101: 1676-1948	**II-3 / Good**
trastuzumab	Seidman, A. D., M. N. Fornier, et al. (2001). "Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification." J Clin Oncol 19(10): 2587-95. 2)	**II-2 / Good**

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FIGURE 16M

LITERATURE LEVEL OF EVIDENCE ASSESSMENT FRAMEWORK

Study Design

Hierarchy of Design	Criteria
I	Evidence obtained from at least one properly designed **randomized controlled trial**.
II-1	Evidence obtained from well-designed controlled trials **without randomization**.
II-2	Evidence obtained from well-designed **cohort** or **case-control** analytic studies, preferably from more than one center or research group.
II-3	Evidence obtained from multiple time series with or without the intervention. Dramatic results in uncontrolled trials might also be regarded as this type of evidence.
III	Opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees.

Study Validity

Grade	Criteria
Good	The study is judged to be valid and relevant as regards results, statistical analysis, and conclusions and shows no significant flaws.
Fair	The study is judged to be valid and relevant as regards results, statistical analysis, and conclusions, but contains at least one significant but not fatal flaw.
Poor	The study is judged to have a fatal flaw such that the conclusions are not valid for the purposes of this test.

* Adapted from Harris, T., D. Atkins, et al. (2001). "Current Methods of the U.S. Preventive Services Task Force." Am J Prev Med 20(3S)[9]

Disclaimer

This test was developed by Caris Life Sciences and their performance characteristic was determined by Caris Life Sciences. It has not been cleared or approved by the U. S. Food and Drug Administration (FDA). These tests are permitted for clinical purposes and should not be regarded as purely investigational or for research only. Caris Life Sciences is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) as qualified to perform high-complexity clinical testing.

By requesting and/or utilizing this test and the report, you agree that the associated analysis, interpretation, and intellectual property generated by the utilization or included in the report is copyright protected, proprietary, and owned by Caris Life Sciences. Caris Life Sciences grants to the physician a limited right to use the information to care for the associated patient, but for no other purpose, including but not limited to validating or creating a similar test, program, or report, which is strictly prohibited unless by the express written permission of Caris Life Sciences and with appropriate patient consents. By requesting and/or utilizing this report, you agree that in the event of a breach of these provisions, Caris Life Sciences shall be entitled to receive as liquidated damages an immediate payment of $100,000 for each violation and 1% of the total amount for each day that such a breach continues while acknowledging and agreeing that actual damages for such a breach would be difficult to calculate, and that such an amount constitutes a reasonable estimate necessary to compensate Caris Life Sciences for the damage suffered by it as a result of the breach of these provisions that are intended to limit the use of the test for the care of the associated patient.

Decisions on care and treatment should be based on the independent medical judgment of the treating physician taking into consideration all available information concerning the patient's condition, including other laboratory tests, in accordance with the standard of care in a given community. Decisions regarding care and treatment should not be based on a single test such as this test. The finding of a biomarker expression does not necessarily indicate pharmacologic effectiveness or lack thereof. If a patient's tumor has previously progressed on an agent identified as associated with clinical benefit on this report, the patient should not be re-treated with this agent.

Not Signed

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FIGURE 16N

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/28801

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/50 (2011.01)
USPC - 436/63, 436/64
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 33/50 (2011.01)
USPC-436/63, 436/64

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google Patents; Google Scholar
sparc, hsp90, cancer or malignan$ or theranos$ or diagnos$, therapy, expression, monoclonal, perovidase, biomarker, interdigitating dendritic cell sarcoma, RNA, protein, microarray, immunohistochemistry, IHC, tumor, elevated, overexpressed, mitosis or mitotic inhibitor,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0117133 A1 (TRIEU et al.) 24 May 2007 (24.05.2007) (para [0002], [0005], [0012]-[0015], [0029]-[0035], [0037], [0043], [0044], [0047], [0057], [0059], [0070], [0071], [0110], [0112], [0136], [0138], [0139], [0141]) 1-54	1-43, 45-54
Y	US 2009/0039811 A1 (CHENE et al.) 12 February 2009 (12.02.2009) (para [0002], [0031]-[0033])	1-43, 45-54
Y	US 2003/0219715 A1 (LAM et al.) 27 November 2003 (27.11.2003) (para [0011], [0067]-[0069], [0125], [0173], [0174])	8, 9
Y	COVELL et al. Linking Tumor Cell Cytotoxicity to Mechanism of Drug Action: An Integrated Analysis of Gene Expression, Small-Molecule Screening and Structural Databases PROTEINS: Structure, Function, and Bioinformatics 59:403?433 (2005) (abstract; Tables III, IV).	21
Y	US 2009/0004691 A1 (ERICKSON et al.) 01 January 2009 (01.01.2009) (para [0011])	54
Y,P	US 2010/0069298 A1 (PENNY et al.) 18 March 2010 (18.03.2010) (para [0006]-[0014], [0035])	1-43, 45-54

☐ 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
28 April 2011 (28.04.2011)	10 MAY 2011

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/28801

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.: 44
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.