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(54) Title: EXPRESSION PROFILE ALGORITHM AND TEST FOR CANCER PROGNOSIS

(57) Abstract: The present invention provides a noninvasive, quantitative test for prognosis determination in cancer patients. The test relies on measurements of the tumor levels of certain messenger RNAs (mRNAs). These mRNA levels are inserted into a polynomial formula (algorithm) that yields a numerical recurrence score, which indicates recurrence risk.

WO 2005/008213 A3

EXPRESSION PROFILE ALGORITHM AND TEST FOR CANCER PROGNOSIS

Field of the Invention

The present invention provides a noninvasive, quantitative test for prognosis determination in cancer patients. The test relies on measurements of the tumor levels of certain messenger RNAs (mRNAs) or the corresponding gene expression products. These mRNA or protein levels are entered into a polynomial formula (algorithm) that yields a numerical score, which indicates recurrence risk (recurrence score) or the likelihood of patient response to therapy (response score).

Description of the Related Art

A need exists for clinical tests that help oncologists make well reasoned treatment decisions. One of the most fundamental decisions an oncologist faces in everyday practice is whether to treat or forego treatment of a particular patient with chemotherapeutic agents. Current therapeutic agents for cancer generally have modest efficacy accompanied by substantial toxicity. Thus, it is highly desirable to predetermine which patients (post-resection of the primary tumor) are likely to have metastatic recurrence. If there were a reliable way to obtain this information, high risk patients could be selected for adjuvant chemotherapy and patients unlikely to have cancer recurrence could be spared unnecessary exposure to the adverse events associated with chemotherapy. Similarly, before subjecting a patient (either before or after resection of a primary tumor) to a particular treatment, it would be desirable to know whether the patient is likely to respond to such treatment. For patients unlikely to respond to a certain treatment modality (e.g. treatment with certain chemotherapeutic agents and/or radiology), other treatments can be designed and used, without wasting valuable time.

Although modern molecular biology and biochemistry have revealed hundreds of genes whose activities influence the behavior and state of differentiation of tumor cells, and their sensitivity or resistance to certain therapeutic drugs, with a few exceptions, the status of these genes has not been exploited for the purpose of routinely making clinical decisions about drug treatments. One notable exception is the use of estrogen receptor (ER) protein expression in breast carcinomas to select patients for treatment with anti-estrogen drugs, such as tamoxifen. Another exceptional example is the use of ErbB2

(Her2) protein expression in breast carcinomas to select patients for treatment with the anti-Her2 antibody, Herceptin® (Genentech, Inc., South San Francisco, CA).

The present invention pertains to cancer, such as breast cancer, the biological understanding of which is still rudimentary. It is clear that the classification of breast cancer into a few subgroups, such as $ErbB2^+$ subgroup, and subgroups characterized by low to absent gene expression of the estrogen receptor (ER) and a few additional factors (Perou *et al., Nature* 406:747-752 (2000) does not reflect the cellular and molecular heterogeneity of breast cancer, and does not permit optimization of patient treatment and care. The same is true for other types of cancers, many of which are much less studied and understood than breast cancer.

Currently, diagnostic tests used in clinical practice are single analyte, and therefore fail to capture the potential value of knowing relationships between dozens of different tumor markers. Moreover, diagnostic tests are frequently not quantitative, relying on immunohistochemistry. This method often yields different results in different laboratories, in part because the reagents are not standardized, and in part because the interpretations are subjective and cannot be easily quantified. RNA-based tests have not often been used because of the problem of RNA degradation over time and the fact that it is difficult to obtain fresh tissue samples from patients for analysis. Fixed paraffin-embedded tissue is more readily available and methods have been established to detect RNA in fixed tissue. However, these methods typically do not allow for the study of large numbers of genes from small amounts of material. Thus, traditionally, fixed tissue has been rarely used other than for immunohistochemistry detection of proteins.

In the past few years, several groups have published studies concerning the classification of various cancer types by microarray gene expression analysis {see, e.g. Golub *et al., Science* 286:531-537 (1999); Bhattacharjae *et al., Proc. Natl. Acad. Sci. USA* 98:13790-13795 (2001); Chen-Hsiang *et al., Bioinformatics* 17 (Suppl. 1):S316-S322 (2001); Ramaswamy *et al., Proc. Natl. Acad. Sci. USA* 98:15149-15154 (2001)). Certain classifications of human breast cancers based on gene expression patterns have also been reported {Martin *et al., Cancer Res.* 60:2232-2238 (2000); West *et al., Proc. Natl. Acad. Sci. USA* 98:11462-11467 (2001)}. Most of these studies focus on improving and refining the already established classification of various types of cancer, including breast cancer. A few studies identify gene expression patterns that may be

2004258085 28 Apr 2010

prognostic {Sorlie *et al., Proc. Natl. Acad. Sci. USA* 98:10869-10874 (2001); Yan *et al., Cancer Res.* 61:8375-8380 (2001); Van De Vivjer *et al. New England Journal of Medicine 347: 1999-2009 (2002)*}, but due to inadequate numbers of screened patients, are not yet sufficiently validated to be widely used clinically.

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Summary of the Invention

A first aspect provides a method for determining the likelihood of breast cancer recurrence or response to hormonal therapy in a mammalian subject comprising:

(a) measuring the expression levels of the RNA transcripts of at least four of the following genes GRB7, HER2, EstR1(ER), PR, Bcl2, CEGP1, SURV, Ki.67, MYBL2, CCNB1, STK15, CTSL2, and STMY3 according to (b), or their expression products in a biological sample comprising a breast tumor cell obtained from said subject;

(b) creating the following gene subsets each comprising an expression level of at least one of said genes or a substitute gene which coexpresses with each of the selected genes with a Pearson correlation coefficient of ≥ 0.40:

(i) a growth factor subset: GRB7 and HER2;

(ii) an estrogen receptor subset: EstR1(ER), PR, Bcl2 and CEGP1;

(iii) a proliferation subset: SURV, Ki.67, MYBL2, CCNB1 and STK15; and

(iv) an invasion subset: CTSL2 and STMY3;

(c) calculating the recurrence score (RS) for said subject by weighting the measured expression levels of each of the gene subsets by contribution to breast cancer recurrence;

(d) using said RS to determine the likelihood of breast cancer recurrence or response to therapy; and

(e) creating a report summarizing the result of said determination.

A second aspect provides a method for determining the likelihood of breast cancer recurrence or response to hormonal therapy in a mammalian subject comprising:

(a) measuring expression levels of RNA transcripts for at least four of a plurality of genes or their expression products, in a biological sample containing tumor cells obtained from said subject, wherein the plurality of genes are grouped in gene subsets comprising:

(i) a growth factor subset;

(ii) an estrogen receptor subset;

(iii) a proliferation subset; and

(iv) an invasion subset,

wherein at least one gene within subset (i) is selected from the group consisting of: GFB7; HER2; Q9BRT3; TCAP; PNMT; ML64; IPPD; Q9H7G1; Q9HBS1; Q9Y220; PSMD3; and CSF3,

wherein at least one gene within subset (ii) is selected from the group consisting of: EstR1(ER); PR; Bcl2; CEGP1; HNF3A; ErbB3; GATA3; BECN1; IGF1R; AKT2; DHPS; BAG1; hENT1; TOP2B; MDM2; CCND1; DKFZp586M0723; NPD009; B.Catenin; IRS1; Bclx; RBM5; PTEN; A.Catenin; KRT18; ZNF217; ITGA7; GSN; MTA1; G.Catenin; DR5; RAD51C; BAD; TP53BP1; RIZ1; IGFBP2; RUNX1; PPM1D; TFF3; S100A8; P28; SFRS5; and IGFBP2,

wherein at least one gene within subset (iii) is selected from the group consisting of: SURV; Ki67; MYBL2; CCNB1; STK15; C20.orf1, TOP2A; CDC20; KNSL2; MELK; TK1; NEK2; LMNB1; PTTG1; BUB1; CCNE2; FLJ20354; MCM2; RAD54L; PRO2000; PCNA; Chk1; NME1; TS; FOXM1; AD024; and HNRPAB, and

wherein at least one gene within subset (iv) is selected from the group consisting of: CTSL2 and STMY3;

wherein a substitute gene for CD68 is selected from the group consisting of: CTSB; CD18; CTSL; HLA.DPB1; and MMP9,

wherein a substitute gene for GSTM1 is selected from the group consisting of: GSTM2; GSTM3; GSTM4; GSTM5; MYH11; GSN; and ID1, and wherein a substitute gene for BAG1 is selected from the group consisting of: Bcl2, GATA3, DHPS, and HNF3A;

(b) calculating a recurrence score (RS) for said subject by weighting the measured expression levels for each of the gene subsets by contribution to breast cancer recurrence;

(c) using the RS to determine a likelihood of breast cancer recurrence or response to therapy;

(d) creating a report based on the likelihood of breast cancer recurrence or response to therapy.

This invention relates to an algorithm-based prognostic test for determining the likelihood of cancer recurrence and/or the likelihood that a patient responds well to a treatment modality. Disclosed herein is an algorithm and prognostic test for determining the likelihood of breast cancer recurrence and/or patient response to treatment, in patients with invasive breast cancer. Hence, the disclosure finds utility in making treatment decisions concerning the therapy of cancer patients, such as, for example, lymph node-negative breast cancer patients who have undergone surgical resection of their tumors. Specifically, the algorithm and related prognostic test helps guide the decision whether to treat such patients with adjunct chemotherapy, and if the answer is affirmative, which treatment option(s) to choose.

Features of the algorithm that distinguish it from other breast cancer prognostic methods include: 1) a unique set of test mRNAs (or the corresponding gene expression products) used to determine recurrence likelihood; 2) certain weights used to combine the expression data into a formula; 3) thresholds used to divide patients into groups of different levels of risk, such as low, medium, and high risk groups. The algorithm yields a numerical recurrence score (RS) or, if patient response to treatment is assessed, response to therapy score (RTS). The test requires a laboratory assay to measure the levels of the specified mRNAs or their expression products, but can utilize very small amounts of either fresh tissue, or frozen tissue or fixed, paraffin-embedded tumor biopsy specimens that have already been necessarily collected from patients and archived. Thus, the test can be noninvasive. It is also compatible with several different methods of tumor tissue harvest, for example, via core biopsy or fine needle aspiration. The tumor tissue can be, but does not have to be, grossly dissected away from normal tissue. Further, for each member of the gene set, the disclosure specifies oligonucleotide sequences that can be used in the test. The mRNA levels are normalized against a specified set of reference genes. The disclosure encompasses all subsequences of the test and reference genes, including 5' and 3' untranslated and intron sequences. Protein levels can be normalized in an analogous manner.

Thus, disclosed herein is a method of classifying a tumor according to the likelihood of cancer recurrence or response to therapy in a mammalian subject, comprising:

(a) subjecting a biological sample comprising cancer cells obtained from said subject to gene or protein expression profiling;

(b) quantifying the gene or protein expression level of multiple individual genes so as to determine an expression value for each gene;

(c) creating subsets of the gene or protein expression values, each subset comprising expression values for genes or proteins linked by a cancer-related biological function and/or by co-expression;

(d) multiplying the expression level of each gene within a subset by a coefficient reflecting its relative contribution to cancer recurrence or response to therapy within said subset and adding the products of multiplication to yield a term for said subset;

(e) multiplying the term of each subset by a factor reflecting its contribution to cancer recurrence or response to therapy; and

(f) producing the sum of terms for each subset multiplied by said factor to produce a recurrence score (RS) or a response to therapy (RTS) score,

wherein the contribution of each subset which does not show a linear correlation with cancer recurrence or response to therapy is included only above a predetermined threshold level, and

wherein the subsets in which increased expression of the genes or proteins included reduce risk of cancer recurrence are assigned a negative value, and the subsets increased expression of the genes or proteins included increase risk of cancer recurrence are assigned a positive value.

The algorithm is suitable to assess the risk of cancer recurrence or response to therapy for any type of cancer, but is exemplified, without limitation, for breast cancer.

In the case of breast cancer, the genes contributing to the recurrence algorithm include 16 test genes and 5 reference genes.

The test genes/mRNAs specified are: Grb7; Her2; ER; PR; BCl2; CEGP1; SURV; Ki-67; MYBL2; CCNB1; STK15; CTSL2; STMY3; GSTM1; BAG1; CD68.

The reference genes/mRNAs are: β-ACTIN; GAPDH; RPLPO; TFRC; GUS.

For breast cancer, the above set of 16 test genes includes 4 multi-gene subsets. The gene members of these subsets have in common both correlated expression and categories of biological function: a proliferation subset comprising SURV, Ki-67, MYBL2, CCNB1 and STK15; a growth factor subset comprising Her2 and Grb7; an estrogen receptor subset comprising ER, PR, BCl2, and CEGP1; an invasion subset comprising genes for the invasive proteases CTSL2 and STMY3.

Thus, disclosed herein is a method for determining the likelihood of breast cancer recurrence or response to hormonal therapy in a mammalian subject comprising:

(a) determining the expression levels of the RNA transcripts of GRB7, HER2, ER, PR, Bcl2, CEGP1, SURV, Ki.67, MYBL2, CCNB1, STK15, CTSL2, and STMY3, or their expression products, or corresponding substitute genes or their expression products, in a biological sample containing tumor cells obtained from said subject;

(b) creating the following gene subsets:

(i) growth factor subset: GRB7 and HER2;

(ii) differentiation (estrogen receptor) subset: ER, PR, Bcl2, and CEGP1;

(iii) proliferation subset: SURV, Ki.67, MYBL2, CCNB1, and STK15; and

(iv) invasion subset: CTSL2, and STMY3;

wherein a gene within any of subsets (i)-(iv) can be substituted by substitute gene which coexpresses with said gene in said tumor with a Pearson correlation coefficient of ≥ 0.40; and

(c) calculating the recurrence score (RS) or response to therapy score (RTS) for said subject by weighting the contributions of each of subsets (i) - (iv), to breast cancer recurrence or response to therapy.

2004258085 28 Apr 2010

The method may further comprise determining the expression levels of RNA transcripts of CD68, GSTM1 and BAG1 or their expression products, or corresponding substitute genes or their expression products, and including the contribution of said genes or substitute genes to breast cancer recurrence or response to therapy in calculating the RS or RTS,

wherein a higher RS or RTS represents an increased likelihood of breast cancer recurrence or lower likelihood of response to therapy, as applicable.

Disclosed herein is a method for determining the likelihood of breast cancer recurrence in a mammalian subject comprising:

(a) determining the expression levels of GRB7, HER2, EstR1, PR, Bcl2, CEGP1, SURV, Ki.67, MYBL2, CCNB1, STK15, CTSL2, STMY3, CD68, GSTM1, and BAG1, or their expression products, in a biological sample containing tumor cells obtained from said subject; and

(b) calculating the recurrence score (RS) by the following equation:

RS = (0.23 to 0.70) x GRB7axisthresh - (0.17 to 0.51) x ERaxis + (0.53 to 1.56) x prolifaxisthresh + (0.07 to 0.21) x invasionaxis + (0.03 to 0.15) x CD68 - (0.04 to 0.25) x GSTM1 - (0.05 to 0.22) x BAG1

wherein

(i) GRB7 axis = (0.45 to 1.35) x GRB7 + (0.05 to 0.15) x HER2;

(ii) if GRB7 axis < -2, then GRB7 axis thresh = -2, and
if GRB7 axis ≥ -2, then GRB7 axis thresh = GRB7 axis;

(iii) ER axis = (Est1 + PR + Bcl2 + CEGP1)/4;

(iv) prolifaxis = (SURV + Ki.67 + MYBL2 + CCNB1 + STK15)/5;

(v) if prolifaxis < -3.5, then prolifaxisthresh = -3.5,
if prolifaxis ≥ -3.5, then prolifaxishresh = prolifaxis; and

(vi) invasionaxis = (CTSL2 + STMY3)/2,

wherein the terms for all individual genes for which ranges are not specifically shown can vary between about 0.5 and 1.5, and wherein a higher RS represents an increased likelihood of breast cancer recurrence.

The laboratory assay for gene expression can use a variety of different platforms. The preferred embodiment for use with fixed paraffin-embedded tissues is quantitative reverse transcriptase polymerase chain reaction qRT-PCR. However, the other technology platforms including mass spectroscopy and DNA microarrays, and can also be used. In the case of DNA microarrays the polynucleotide probes can be cDNAs ("cDNA arrays") that are typically about 500 to 5000 bases long, although shorter or longer cDNAs can also be used and are within the scope of this invention. Alternatively, the polynucleotides can be oligonucleotides (DNA microarrays), which are typically about 20 to 80 bases long, although shorter and longer oligonucleotides are also suitable and are within the scope of the invention. The solid surface can, for example, be glass or nylon, or any other solid surface typically used in preparing arrays, such as microarrays, and is typically glass.

Brief Description of the Drawings

Table 1 lists test and reference genes, expression of which is used to construct the breast cancer recurrence algorithm. The table includes accession numbers for the genes, sequences for the forward and reverse primers (designated by "f" and "r", respectively) and probes (designated by "p") used for PCR amplification.

Table 2 shows the amplicon sequences of the aforementioned genes.

Figures 1A and B present results from a clinical study of 242 patients with invasive, lymph node-negative breast cancer, for whom 10 year recurrence data were available. The Figure 1A presents proportion of patients with actual cancer recurrence (Kaplan-Meier method) versus calculated recurrence scores. Patients are grouped in 5 percentile units, except for patients with scores <-4.5, who are all grouped together. Figure 1B is similar to Figure 1A. It also presents proportion of patients with actual cancer recurrence, but in this case the actual recurrence data are plotted against percentiles of recurrence scores. Patients are grouped in 5 percentile units, except for patients in the lower 20 percentile, who are all grouped together.

Figure 2 illustrates the relationship between recurrence score {RS} and rate or likelihood of distant recurrence of breast cancer in 10 years. Data are taken from the NSABP B-14 patient population. Broken lines represent 95% confidence intervals. Each tick mark on the X-axis denotes a patient having the corresponding RS.

2004258085 28 Apr 2010

Detailed Description of the Preferred Embodiment

A. Definitions

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, NY 1994), and March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, NY 1992), provide one skilled in the art with a general guide to many of the terms used in the present application.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

The term "microarray" refers to an ordered arrangement of hybridizable array elements, preferably polynucleotide probes, on a substrate.

The term "polynucleotide," when used in singular or plural, generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The term "oligonucleotide" refers to a relatively short polynucleotide, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including *in vitro* recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

The terms “differentially expressed gene,” “differential gene expression” and their synonyms, which are used interchangeably, refer to a gene whose expression is activated to a higher or lower level in a subject suffering from a disease, specifically cancer, such as breast cancer, relative to its expression in a normal or control subject. The terms also include genes whose expression is activated to a higher or lower level at different stages of the same disease. It is also understood that a differentially expressed gene may be either activated or inhibited at the nucleic acid level or protein level, or may be subject to alternative splicing to result in a different polypeptide product. Such differences may be evidenced by a change in mRNA levels, surface expression, secretion or other partitioning of a polypeptide, for example. Differential gene expression may include a comparison of expression between two or more genes or their gene products, or a comparison of the ratios of the expression between two or more genes or their gene products, or even a comparison of two differently processed products of the same gene, which differ between normal subjects and subjects suffering from a disease, specifically cancer, or between various stages of the same disease. Differential expression includes both quantitative, as well as qualitative, differences in the temporal or cellular expression pattern in a gene or its expression products among, for example, normal and diseased cells, or among cells which have undergone different disease events or disease stages. For the purpose of this invention, “differential gene expression” is considered to be present when there is at least an about two-fold, preferably at least about four-fold, more preferably at least about six-fold, most preferably at least about ten-fold difference between the expression of a given gene in normal and diseased subjects, or in various stages of disease development in a diseased subject.

The phrase "gene amplification" refers to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA (mRNA) produced, *i.e.,* the level of gene expression, also increases in proportion to the number of copies made of the particular gene expressed.

The term "prognosis" is used herein to refer to the prediction of the likelihood of cancer-attributable death or progression, including recurrence, metastatic spread, and drug resistance, of a neoplastic disease, such as breast cancer.

The term "prediction" is used herein to refer to the likelihood that a patient will respond either favorably or unfavorably to a drug or set of drugs, and also the extent of those responses, or that a patient will survive, following surgical removal or the primary tumor and/or chemotherapy for a certain period of time without cancer recurrence. The predictive methods of the present invention are valuable tools in predicting if a patient is likely to respond favorably to a treatment regimen, such as surgical intervention, chemotherapy with a given drug or drug combination, and/or radiation therapy, or whether long-term survival of the patient, following surgery and/or termination of chemotherapy or other treatment modalities is likely.

The term "long-term" survival is used herein to refer to survival for at least 5 years, more preferably for at least 8 years, most preferably for at least 10 years following surgery or other treatment.

The term "tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

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

The "pathology" of (tumor) cancer includes all phenomena that compromise the well-being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, typically: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent that those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

In the context of the present invention, reference to "at least one," "at least two," "at least five," etc. of the genes listed in any particular gene set means any one or any and all combinations of the genes listed.

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

The term "gene expression profiling" is used in the broadest sense, and includes methods of quantification of mRNA and/or protein levels in a biological sample.

The term "adjuvant therapy" is generally used to treatment that is given in addition to a primary (initial) treatment. In cancer treatment, the term "adjuvant therapy" is used to refer to chemotherapy, hormonal therapy and/or radiation therapy following surgical removal of tumor, with the primary goal of reducing the risk of cancer recurrence.

"Neoadjuvant therapy" is adjunctive or adjuvant therapy given prior to the primary (main) therapy. Neoadjuvant therapy includes, for example, chemotherapy, radiation therapy, and hormone therapy. Thus, chemotherapy may be administered prior to surgery to shrink the tumor, so that surgery can be more effective, or, in the case of previously unoperable tumors, possible.

The term "cancer-related biological function" is used herein to refer to a molecular activity that impacts cancer success against the host, including, without limitation, activities regulating cell proliferation, programmed cell death (apoptosis), differentiation, invasion, metastasis, tumor suppression, susceptibility to immune surveillance, angiogenesis, maintenance or acquisition of immortality.

B. Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", 2nd edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology", 4th edition (D.M. Weir & C.C. Blackwell, eds., Blackwell Science Inc., 1987); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); and "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

Disclosed herein is an algorithm for determining the likelihood of cancer recurrence or response to therapy in cancer patients. The method is based on (1) the identification and clustering of a set of genes that can serve as markers of cancer recurrence or the likelihood of patient response to a particular therapy, (2) certain weights assigned to the clusters and the individual genes within the clusters that reflect their value in predicting cancer recurrence or response to therapy and used to combine the expression data into a formula; and (3) determination of threshold values used to divide patients into groups with varying degrees of risk of cancer recurrence, or with varying likelihood of response to treatment, such as low, medium, and high risk groups or groups in which the likelihood of patient response to a particular treatment is low, medium or high. The algorithm yields a numerical recurrence score (RS) or response to treatment score (RTS), which can be used to make treatment decisions concerning the therapy of cancer patients.

The first step in generating data to be analyzed by the algorithm of the present invention is gene or protein expression profiling.

1. *Techniques of Expression Profiling*

The present disclosure requires an assay to measure the levels of specified genes (mRNAs) or their expression products (proteins) in a biological sample comprising cancer cells. Typically, the biological sample is a fresh or archived tissue sample obtained from a tumor, e.g. by tumor biopsy or aspiration, but biological fluids containing tumor cells can also be used in the analysis.

In its most common form, gene expression profiling involves the determination of mRNA levels in a tissue sample, such as a fixed, paraffin-embedded tumor biopsy specimen that has already been collected from the patient and archived. Thus, the test can be completely non-invasive. It is also compatible with several different methods of tumor tissue harvest, for example, core biopsy and fine needle aspiration. The tumor tissue can be, but does not need to be, grossly dissected away from normal tissue.

Methods of gene expression profiling directed to measuring mRNA levels can be divided into two large groups: methods based on hybridization analysis of polynucleotides, and methods based on sequencing of polynucleotides. The most 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, *Methods in Molecular Biology* 106:247-283 (1999)); RNAse protection assays (Hod, *Biotechniques* 13:852-854 (1992)); and reverse transcription polymerase chain reaction (RT-PCR) (Weis *et al., Trends in Genetics* 8:263-264 (1992)). 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).

In all of these techniques, the first step is the isolation of mRNA 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, including breast, lung, colon, prostate, brain, liver, kidney, pancreas, spleen, thymus, testis, ovary, uterus, etc., tumor, or tumor cell lines, 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.

General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., Current Protocols of Molecular Biology, John Wiley and Sons (1997). Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, Lab Invest. 56:A67 (1987), and De Andrés 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. Other commercially available RNA isolation kits include MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, WI), and Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be isolated using RNA Stat-60 (Tel-Test). RNA prepared from tumor can be isolated, for example, by cesium chloride density gradient centrifugation.

While the practice of the invention will be illustrated with reference to techniques developed to determine mRNA levels in a biological (e.g. tissue) sample, other techniques, such as methods of proteomics analysis are also included within the broad concept of gene expression profiling, and are within the scope herein. In general, a preferred gene expression profiling method for use with paraffin-embedded tissue is quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), however, other technology platforms, including mass spectroscopy and DNA microarrays can also be used.

In the following sections, a series of representative, but not exhaustive, gene expression profiling techniques will be discussed in greater detail.

2. *Reverse Transcriptase PCR (RT-PCR)*

Of the techniques listed above, the most sensitive and most flexible quantitative method is qRT-PCR, which can be used to compare mRNA levels 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 mRNAs, and to analyze RNA structure.

As RNA cannot serve as a template for PCR, the first step in gene expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The two most commonly used reverse transcriptases are 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, CA, USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

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. Thus, 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.

TaqMan® RT-PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700TM Sequence Detection SystemTM (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA), or Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). In a preferred embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700TM Sequence Detection SystemTM. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system includes software for running the instrument and for analyzing the data.

5'-Nuclease assay 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).

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.

A more recent variation of the RT-PCR technique is the real time quantitative PCR, which measures 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. For further details see, e.g. Held et al., Genome Research 6:986-994 (1996).

3. *Microarrays*

Differential gene expression can also be identified, or confirmed using the microarray technique. Thus, the expression profile of breast cancer-associated genes can be measured in either fresh or paraffin-embedded tumor tissue, using microarray technology. In this method, polynucleotide sequences of interest (including cDNAs and oligonucleotides) are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific DNA probes from cells or tissues of interest. Just as in the RT-PCR method, the source of mRNA typically is total RNA isolated from 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.

In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate in a dense array. Preferably at least 10,000 nucleotide sequences are applied to the substrate. The microarrayed genes, immobilized on the microchip at 10,000 elements each, 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., Proc. Natl. Acad. Sci. USA 93(2):106-149 (1996)). Microarray analysis can be performed by commercially available equipment, following manufacturer's protocols, such as by using the Affymetrix GenChip technology, or Incyte's microarray technology.

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.

4. *Serial Analysis of Gene Expression (SAGE)*

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 (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. For more details see, e.g. Velculescu *et al., Science* 270:484-487 (1995); and Velculescu *et al., Cell* 88:243-51 (1997).

5. *Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS)*

This method, described by Brenner *et al., Nature Biotechnology* 18:630-634 (2000), is a sequencing approach that combines non-gel-based signature sequencing with *in vitro* cloning of millions of templates on separate 5 μm diameter 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 (typically greater than 3 x 10^6 microbeads/cm^2). 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 yeast cDNA library.

6. *Immunohistochemistry*

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

7. *Proteomics*

The term "proteome" is defined as the totality of the proteins present in a sample (e.g. tissue, organism, or cell culture) at a certain point of time. Proteomics includes, among other things, study of the global changes of protein expression in a sample (also referred to as "expression proteomics"). Proteomics typically includes the following steps: (1) separation of individual proteins in a sample by 2-D gel electrophoresis (2-D PAGE); (2) identification of the individual proteins recovered from the gel, e.g. my mass

2004258085 28 Apr 2010

spectrometry or N-terminal sequencing, and (3) analysis of the data using bioinformatics. Proteomics methods are valuable supplements to other methods of gene expression profiling, and can be used, alone or in combination with other methods, to detect the products of the prognostic markers of the present disclosure.

8. *Cancer Gene Set, Assayed Gene Subsequences and Clinical Application of Gene Expression Data*

It is important to the present disclosure to use the measured expression of certain genes, or their expression products, by cancer, e.g. breast cancer, tissue to provide prognostic information. For this purpose, it is necessary to correct for (normalize away) both differences in the amount of RNA assayed and variability in the quality of the RNA used. Therefore, the assay typically measures and incorporates the expression of certain normalizing genes, including well know housekeeping genes, such as GAPDH and β-ACTIN. Alternatively, normalization can be based on the mean or median signal (Ct) of all of the assayed genes or a large subset thereof (global normalization approach). Throughout the disclosure, unless noted otherwise, reference to expression levels of a gene assumes normalized expression relative to the reference set although this is not always explicitly stated.

9. *Algorithm to Generate a Cancer Recurrence Score*

When qRT-PCR is used to measure mRNA levels, mRNA amounts are expressed in Ct (threshold cycle) units (Held *et al., Genome Research* 6:986-994 (1996)). The averaged sum of reference mRNA Cts is set as zero, and each measured test mRNA Ct is given relative to this zero point. For example, if, for a certain patient tumor specimen the average of Cts of the 5 reference genes found to be 31 and Ct of the test gene CRB7 is found to be 35, the reported value for GRB7 is -4 (i.e. 31-35).

As a first step following the quantitative determination of mRNA levels, the genes identified in the tumor specimen and known to be associated with the molecular pathology of cancer are grouped into subsets. Thus, genes known to be associated with proliferation will constitute the "proliferation subset" (axis). Genes known to be associated with invasion of cancer will constitute the "invasion subset" (axis). Genes associated with key growth factor receptor pathway(s) will constitute the "growth factor subset" (axis). Genes known to be involved with activating or signaling through the estrogen receptor (ER) will constitute the "estrogen receptor subset" (axis). This list of subsets is, of course, not limiting. The subsets (axes) created will depend on the particular cancer, i.e. breast, prostate, pancreatic, lung, etc. cancer. In general, genes the expression of which is known to correlate with each other, or which are known to be involved in the same pathway are grouped in the same axis.

In the next step, the measured tumor level of each mRNA in a subset is multiplied by a coefficient reflecting its relative intra-set contribution to the risk of cancer recurrence and this product is added to the other products between mRNA levels in the subset and their coefficients, to yield a term, e.g. a proliferation term, an invasion term, a growth factor term, etc. For example, in the case of lymph node-negative invasive breast cancer the growth factor term is (0.45 to 1.35) x GRB7 + (0.05 to 0.15) x Her2 (see the Example below).

The contribution of each term to the overall recurrence score is weighted by use of a coefficient. For example, in the case of lymph node-negative invasive breast cancer the coefficient of the growth factor term can be between 0.23 and 0.70.

Additionally, for some terms, such as the growth factor and proliferation terms, a further step is performed. If the relationship between the term and the risk of recurrence is non-linear, a non-linear functional transform of the term, such as a threshold is used Thus, in lymph node-negative invasive breast cancer, when the growth factor term is found at <-2 the value is fixed at -2. When the proliferation term is found at <-3.5 the value is fixed at-3.5.

The sum of the terms obtained provides the recurrence score (RS).

A relationship between recurrence score (RS) and risk of recurrence has been found by measuring expression of the test and reference genes in biopsied tumor specimens from a population of lymph node-negative patients with invasive breast cancer and applying the algorithm.

It is noted that the RS scale generated by the algorithm of the present invention can be adjusted in various ways. Thus, while the RS scale specifically described above effectively runs from -4.5 to -2.0, the range could be selected such that the scale run from 0 to 10, 0 to 50, or 0 to 100, for example.

For example, in a particular scaling approach, scaled recurrence score (SRS) is calculated on a scale of 0 to 100. For convenience, 10 Ct units are added to each measured Ct value, and unscaled RS is calculated as described before. Scaled recurrence scores (SRS) are calculated using the equations shown below.

$$\text{GRB7 Score} = 0.9 \times \text{GRB7} + 0.1 \times \text{HER2}$$

$$\text{GRB7 Threshold Score} = \begin{cases} 8 & \text{if GRB7 Score} < 8 \\ \text{GRB7 Score} & \text{otherwise} \end{cases}$$

$$\text{ER Score} = \left(0.8 \times \text{Esrt1} + 1.2 \times \text{PR} + \text{Bcl2} + \text{CEGP1}\right)/4$$

$$\text{Proliferation Score} = \left(\text{SURV} + \text{Ki-67} + \text{MYBL2} + \text{CCNB1} + \text{STK15}\right)/5$$

$$\text{Prolif. Threshold Score} = \begin{cases} 6.5 & \text{if Proliferation Score} < 6.5 \\ \text{Proliferation Score} & \text{otherwise} \end{cases}$$

$$\text{Invasion Score} = \left(\text{CTSL2} + \text{STYM3}\right)/2$$

$$\text{SRS} = \begin{cases} 0 & \text{if } 20\times(\text{RS} - 6.7) < 0 \\ 100 & \text{if } 20\times(\text{RS}-6.7) > 100 \\ 20\times(\text{RS}-6.7) & \text{otherwise} \end{cases}$$

where

$$\begin{aligned} \text{RS} = {} & 0.47 \times \text{GRB7 Group Threshold Score} \\ & -0.34 \times \text{ER Group Score} \\ & +1.04 \times \text{Proliferation Group Threshold Score} \\ & +0.10 \times \text{Invasion Group Score} \\ & +0.05 \times \text{CD68} \\ & -0.08 \times \text{GSTM1} \\ & -0.07 \times \text{BAG1 old Score} \end{aligned}$$

Patients assigned to various risk categories using the following recurrence scores:

Risk Category	Recurrence Score
Low Risk of Recurrence	RS < 18
Intermediate Risk	18 ≤RS < 31
High Risk of Recurrence	RS ≥31

This scaled approach was used to calculated the recurrence scores shown in Figure 2.

10. *Use the Recurrence and Response to Treatment Scores*

Recurrence scores (RSs) or response to treatment scores (RTS), as determined by the algorithm of the present invention, provide valuable tools for the practicing physician to make critical treatment decisions. Thus, if the RS of a particular patient is low, the physician might decide that chemotherapy following surgical removal of cancer, e.g. breast cancer is not necessary in order to ensure long term survival of patient. As a result, the patient will not be subject to the often very severe side effects of standard of care chemotherapeutic treatment. If, on the other hand, the RS is determined to be high, this information can be used to decide which chemotherapeutic or other treatment option (such as, radiation therapy) is most suitable for treating the patient. Similarly, if the RTS score of a patient for a particular treatment is low, other, more effective, treatment modalities will be used to combat cancer in that particular patient.

By way of example, current standard of care chemotherapeutic options for the treatment of breast cancer include the administration of anthracyclines plus cyclophosphamide (AC); or AC plus a taxanes (ACT); or C plus methotrexate plus 5-fluorouracil (CMF); or the administration of any of these drugs alone, or in any combination. Other chemotherapeutic drugs often used in cancer, e.g. breast cancer, treatment include, for example, Herceptin®, vinblastine, actinomycin D, etoposide, cisplatin, and doxorubicin. Determining the RS for a particular patient by the algorithm of the present invention will enable the physician to tailor the treatment of the patient such that the patient has the best chance of long term survival while unwanted side-effects are minimized.

Thus, patients with a low risk of cancer recurrence are typically assigned to hormonal treatment alone, or hormonal treatment and a less toxic chemotherapeutic treatment regiment, e.g. using Herceptin®. On the other hand, patients whose risk of cancer recurrence ha been determined to be intermediate or high are typically subjected to more aggressive chemotherapy, such as, for example, anthracycline and/or taxane-based treatment regimens.

The RS value can also be used to decide whether to treat the patient with therapeutic drugs that are currently not part of the main line treatment protocol for a particular cancer, such as, for example, EGFR inhibitors, and/or by other treatment options, such as radiation therapy alone, or before or after chemotherapeutic treatment.

Further details of the invention will be provided in the following non-limiting Examples.

Example 1

Algorithm to Predict Breast Cancer Recurrence

The algorithm is based on measurements of the tumor levels of 16 mRNA markers, which were selected by two major criteria: 1) univariate correlation with breast cancer recurrence in the clinical study described below, and 2) implication in tumor pathology as indicated by published scientific literature.

The selected markers indicate roles of several cellular behaviors and pathways in cancer recurrence: e.g., Her2 growth factor; estrogen receptor, proliferation, and invasion. Consistent with current understanding of the molecular pathology of breast cancer, increased levels of mRNAs representing genes in the growth factor, proliferation, and invasion axes correlate with increased risk of recurrence whereas increased levels of mRNAs representing genes in the estrogen receptor axis correlate with reduced risk of recurrence. Relevant genes in these pathways are not only linked by biological function, but also linked by co-expression, as indicated by Pearson correlation coefficients (data not shown). For calculation of Pearson correlation coefficient see, e.g. K. Pearson and A. Lee, *Biometrika* 2:357 (1902).

An algorithm was constructed by weighing the contributions of certain co-expressed genes in the above pathways. The model was iteratively optimized by selection for best correlation to recurrence risk. Model fitting included selection of best genes representing the above functional and co-expression axes and selection for optimal equation coefficients.

In addition, other genes were included that were not tightly correlated in expression to any of the above genes or each other, but which were also found to independently contribute to prediction of cancer recurrence. In view of accumulating evidence in the biomedical literature that tumor macrophages confer poor prognosis in cancer (M. Orre and P.A Rogers *Gynecol. Oncol.* 73: 47-50 [1999]; L.M. Coussens *et al. Cell* 103: 481-90 [2000]; S. Huang *et al. J. Natl. Cancer Inst.* 94:1134-42 [2002]; M.A. Cobleigh *et al. Proceedings of A.S.C.O.* 22: Abstract 3415 [2003]), the macrophage marker CD68 was also included in the test gene set. Ultimately, a model was identified that included the genes: Grb7; Her2; ER; PR; BCl2; CEGP1; SURV; Ki-67; MYBL2; CCNB1; STK15; CTSL2; STMY3; GSTM1; BAG1; CD68.

The algorithm provides a recurrence risk Score (RS). Specifically, RS is derived as follows:

1. Define the GRB7 axis
   GRB7axis = (0.45 to 1.35) x GRB7 + (0.05 to 0.15) x HER2

2. Define the GRB7 axis threshold
   if GRB7axis < -2
   then GRB7GTthresh = -2,
   else GRB7GTthresh = GRB7axis

3. Define the ER axis
   Eraxis = (EstR1 + PR + Bcl2 + CEGP1)/4, where the individual contributions of the genes listed can be weighted by a factor ranging between 0.5 and 1.5, inclusive.

4. Define the proliferation axis, prolifaxis
   prolifaxis = (SURV + Ki.67 + MYBL2 + CCNB1 + STK15)/5, where the individual contributions of the genes listed can be weighted by a factor between 0.5 and 1.5, inclusive.

5. Define the proliferation axis threshold, prolifaxisthresh
if prolifaxis < -3.5
then prolifaxisthresh = -3.5,
else prolifaxisthresh = prolifaxis

6. Define the invasion axis
invasionaxis = (CTSL2 + STMY3)/2, where the individual contributions of the genes listed can be weighted by a factor between 0.5 and 1.5, inclusive.

7. Calculate the recurrence score (RS)
RS= (0.23 to 0.70) x GRB7GTthresh - (0.17 to 0.51) x ERaxis + (0.52 to 1.56) x prolifaxisthresh + (0.07 to 0.21) x invasionaxis + (0.03 to 0.15) x CD68 - (0.04 to 0.25) x GSTM1 - (0.05 to 0.22) x BAG1.

In a particular embodiment, RS is calculated as follows:

RS = 0.47 x GRB7GTthresh - 0.34 x ERaxis + 1.04 x profilaxisthresh + 0.14 x invasionaxis + 0.11 x CD68 - 0.17 x GSTM1 - 0.15 BAG1.

One skilled in the art will understand that other genes can be substituted for the 16 test genes specified above. Broadly, any gene that co-expresses with a gene in the 16 gene test panel, with Pearson correlation coefficient ≥ 0.40 can be substituted for that gene in the algorithm. To replace gene X by the correlated gene Y, one determines the range of values for gene X in the patient population, the range of values for gene Y in the patient population, and applies a linear transform to map the values for gene Y into the corresponding values for gene X. For example, if the range of values for gene X in the patient population is 0 to 10, and the range of values for gene Y in the patient population is 0 to 5, the appropriate transform is to multiply values of gene Y by 2.

By way of example, the following genes that lie within the Her2 amplicon, are some of the genes that can be substituted into the growth factor gene subset: Q9BRT3; TCAP; PNMT; ML64; IPPD; Q9H7G1; Q9HBS1; Q9Y220; PSMD3; and CSF3

By way of example, some of the genes that can be substituted into the proliferation gene subset are : C20.orf1, TOP2A, CDC20, KNSL2, MELK, TK1, NEK2,

LMNB1, PTTG1, BUB1, CCNE2, FLJ20354, MCM2, RAD54L, PRO2000, PCNA, Chk1, NME1, TS, FOXM1, AD024, and HNRPAB.

By way of example, some of the genes that can be substituted into the estrogen receptor (ER) subset are: HNF3A, ErbB3, GATA3, BECN1, IGF1R, AKT2, DHPS, BAG1, hENT1, TOP2B, MDM2, CCND1, DKFZp586M0723, NPD009, B.Catenin, IRS1, Bclx, RBM5, PTEN, A.Catenin, KRT18, ZNF217, ITGA7, GSN, MTA1, G.Catenin, DR5, RAD51C, BAD, TP53BP1, RIZ1, IGFBP2, RUNX1, PPM1D, TFF3, S100A8, P28, SFRS5, and IGFBP2.

By way of example, some of the genes that can be substituted into the invasion axis are: upa, COL1A1, COL1A2, and TIMP2.

Some of the genes that can be substituted for CD68 are: CTSB, CD18, CTSL, HLA.DPB1, MMP9.

Some of the genes that can be substituted for GSTM1 are: GSTM3.2, MYH11.1, GSN.3, ID1.1.

Some of the genes that can be substituted for BAG1 are: Bcl2.2, GATA3.3, DHPS.3, HNF3A.1.

The RS values determined can be used guide the binary (yes or no) decision whether to treat lymph node negative breast cancer patients with adjuvant chemotherapy. For this purpose a threshold can be defined separating high from low risk patients. As noted before, it may be more informative to define three categories: high, moderate and low risk, which requires two RS cut points. Cut points can be selected to subgroup patients in desired categories of risk by applying the algorithm to certain clinical study data. For example, oncologists may wish to avoid treating lymph node-negative breast cancer patients who carry a less than 10% risk of recurrence within ten years. Application of the algorithm to the clinical trial data described in Example 2 indicates that patients with RS<-3.9 have less than 10% risk of recurrence in ten years, whereas patients with RS>-3.5 have a greater than 39% risk of recurrence in ten years. Patients with intermediate RS values can be considered in the moderate risk category. Oncologists can be provided with RS results in a continuous figure relating recurrence score to ten year Kaplan Meier survival.

Example 2

Study of Gene Expression in 242 Malignant Breast Tumors

A gene expression study was designed and conducted to determine recurrence score (RS) values in a population of patients with node-negative invasive breast cancer and explore the correlation between RS values and disease-free survival. The study utilized archived fixed paraffin-embedded tumor blocks as a source of RNA and matched archived patient records.

Study design:

Molecular assays were performed on paraffin-embedded, formalin-fixed primary breast tumor tissues obtained from 252 individual patients diagnosed with invasive breast cancer. All patients were lymph node-negative, ER-positive, and treated with Tamoxifen. Mean age was 52 years, and mean clinical tumor size was 2 cm. Median follow-up was 10.9 years. As of January 1, 2003, 41 patients had local or distant disease recurrence or breast cancer death. Patients were included in the study only if histopathologic assessment, performed as described in the Materials and Methods section, indicated adequate amounts of tumor tissue and homogeneous pathology.

Materials and Methods:

Each representative tumor block was characterized by standard histopathology for diagnosis, semi-quantitative assessment of amount of tumor, and tumor grade. When tumor area was less than 70% of the section the tumor area was grossly dissected and tissue was taken from 6 (10 micron) sections. Otherwise, a total of 3 sections (also 10 microns in thickness each) were prepared. Sections were placed in two Costar Brand Microcentrifuge Tubes (Polypropylene, 1.7 mL tubes, clear). If more than one tumor block was obtained as part of the surgical procedure, the block most representative of the pathology was used for analysis. Gene Expression Analysis; mRNA was extracted and purified from fixed, paraffin-embedded tissue samples, and prepared for gene expression analysis as described above. Molecular assays of quantitative gene expression were performed by RT-PCR, using the ABI PRISM 7900™ Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA). ABI PRISM 7900™ consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 384-well format on a thermocycler. The system includes software for running the instrument and for analyzing the data.

Results:

Normalized gene expression values were obtained from the above patient specimens and used to calculate RS for each patient, which was matched with respective Kaplan-Meier 10 year survival data as shown in Figures 1A and B. As shown, patients with RS values less than approximately -3.75 have ~90% chance of 10 year recurrence-free survival. Recurrence rates rise sharply at RS values >-3.3. At RS ~ -3.0 risk of recurrence in 10 years is about 30%, and at RS ~ -2.0 risk of recurrence in 10 years is >50%. Figure 1B reveals that approximately 70% of patients are in the lowest risk category.

The results thus demonstrate a close correspondence between RS values as determined by the described test and algorithm and the risk of breast cancer recurrence.

Example 3

Validation of the algorithm of the invention

A prospective clinical validation study was conducted to examine the performance of a multi-gene RT-PCR assay for extracting and quantifying RNA from fixed paraffin embedded tumor tissue in patients enrolled in the tamoxifen alone arm of the National Surgical Adjuvant Breast and Bowel Project (NSABP)

Study B-14: A Laboratory Study To Clinically Validate Whether Genomic Tumor Expression Profiles Can Define The Likelihood Of Recurrence In Patients With Primary Invasive Breast Cancer, Negative Axillary Nodes And Estrogen-Receptor Positive Tumors. The NSABP Study B-14 was conducted to assess the efficacy of post-mastectomy tamoxifen treatment in node-negative primary breast cancer patients and included a total of 2,892 patients who qualified and were treated for five years. The protocol was carried out in a randomized, double-blind manner with one set of patients receiving tamoxifen at a dose of 10 mg twice a day and a control set of patients receiving physically identical placebo tablets. Comparisons of the multi-gene assay to standard histopathologic and molecular measures of tumor classification were performed.

Patient specific recurrence scores derived from the multi-gene assay were obtained for a total of 668 eligible patients from the tamoxifen alone arm of the NSABP Study B-14 (median follow-up time, 14.1 years). Statistical analyses revealed that the resulting patient specific recurrence scores (Figure 2) are significantly (P = 6.5 E-9) correlated to the likelihood of distant recurrence and provide significant information beyond standard clinical measures of tumor classification, including patient age at surgery, clinical tumor size and tumor grade.

It was found that the assay and algorithm of the present invention provide reproducible and informative measures of the likelihood of distant recurrence from tumor tissue collected at the time of surgery for node-negative, ER+, primary breast cancer patients treated with tamoxifen. The patient recurrence score generated by the method of the present invention provides significant ($P < 0.0001$) information beyond standard histopathologic and molecular measures of tumor classification commonly used in clinical practice, including age, clinical tumor size and tumor grade. In contrast to other commonly clinical measures, the patient recurrence score is highly reproducible and unlike other molecular tests simultaneously leverages information across multiple genomic markers, including ER, PR and HER2.

While the present invention has been described with reference to what are considered to be the specific embodiments, it is to be understood that the invention is not limited to such embodiments. To the contrary, the invention is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims. For example, while the disclosure focuses on the identification of various breast cancer associated genes and gene sets, and on the personalized prognosis of breast cancer, similar genes, gene sets and methods concerning other types of cancer are specifically within the scope herein.

All references cited throughout the disclosure are hereby expressly incorporated by reference.

Gene	Accession No.	Name	Artificial Sequence PCR Primer-Probe	Sequence	Length
B-actin	NM_001101	S0034/B-acti.f2	SEQ ID NO: 1	CAGCAGATGTGGATCAGCAAG	21
B-actin	NM_001101	S0036/B-acti.r2	SEQ ID NO: 2	GCATTTGCGGTGGACGAT	18
B-actin	NM_001101	S4730/B-acti.p2	SEQ ID NO: 3	AGGAGTATGACGAGTCCGGCCCC	23
BAG1	NM_004323	S1386/BAG1.f2	SEQ ID NO: 4	CGTTGTCAGCACTTGGAATACAA	23
BAG1	NM_004323	S1387/BAG1.r2	SEQ ID NO: 5	GTTCAACCTCTTCCTGTGGACTGT	24
BAG1	NM_004323	S4731/BAG1.p2	SEQ ID NO: 6	CCCAATTAACATGACCCGGCAACCAT	26
Bcl2	NM_000633	S0043/Bcl2.f2	SEQ ID NO: 7	CAGATGGACCTAGTACCCACTGAGA	25
Bcl2	NM_000633	S0045/Bcl2.r2	SEQ ID NO: 8	CCTATGATTTAAGGGCATTTTTCC	24
Bcl2	NM_000633	S4732/Bcl2.p2	SEQ ID NO: 9	TTCCACGCCGAAGGACAGCGAT	22
CCNB1	NM_031966	S1720/CCNB1.f2	SEQ ID NO: 10	TTCAGGTTGTTGCAGGAGAC	20
CCNB1	NM_031966	S1721/CCNB1.r2	SEQ ID NO: 11	CATCTTCTTGGGCACACAAT	20
CCNB1	NM_031966	S4733/CCNB1.p2	SEQ ID NO: 12	TGTCTCCATTATTGATCGGTTCATGCA	27
CD68	NM_001251	S0067/CD68.f2	SEQ ID NO: 13	TGGTTCCCAGCCCTGTGT	18
CD68	NM_001251	S0069/CD68.r2	SEQ ID NO: 14	CTCCTCCACCCTGGGTTGT	19
CD68	NM_001251	S4734/CD68.p2	SEQ ID NO: 15	CTCCAAGCCCAGATTCAGATTCGAGTCA	28
CEGP1	NM_020974	S1494/CEGP1.f2	SEQ ID NO: 16	TGACAATCAGCACACCTGCAT	21
CEGP1	NM_020974	S1495/CEGP1.r2	SEQ ID NO: 17	TGTGACTACAGCCGTGATCCTTA	23
CEGP1	NM_020974	S4735/CEGP1.p2	SEQ ID NO: 18	CAGGCCCTCTTCCGAGCGGT	20
CTSL2	NM_001333	S4354/CTSL2.f1	SEQ ID NO: 19	TGTCTCACTGAGCGAGCAGAA	21
CTSL2	NM_001333	S4355/CTSL2.r1	SEQ ID NO: 20	ACCATTGCAGCCCTGATTG	19
CTSL2	NM_001333	S4356/CTSL2.p1	SEQ ID NO: 21	CTTGAGGACGCGAACAGTCCACCA	24
EstR1	NM_000125	S0115/EstR1.f1	SEQ ID NO: 22	CGTGGTGCCCCTCTATGAC	19
EstR1	NM_000125	S0117/EstR1.r1	SEQ ID NO: 23	GGCTAGTGGGCGCATGTAG	19
EstR1	NM_000125	S4737/EstR1.p1	SEQ ID NO: 24	CTGGAGATGCTGGACGCCC	19
GAPDH	NM_002046	S0374/GAPDH.f1	SEQ ID NO: 25	ATTCCACCCATGGCAAATTC	20
GAPDH	NM_002046	S0375/GAPDH.r1	SEQ ID NO: 26	GATGGGATTTCCATTGATGACA	22
GAPDH	NM_002046	S4738/GAPDH.p1	SEQ ID NO: 27	CCGTTCTCAGCCTTGACGGTGC	22
GRB7	NM_005310	S0130/GRB7.f2	SEQ ID NO: 28	ccatctgcatccatcttgtt	20
GRB7	NM_005310	S0132/GRB7.r2	SEQ ID NO: 29	ggccaccagggtattatctg	20
GRB7	NM_005310	S4726/GRB7.p2	SEQ ID NO: 30	ctccccacccttgagaagtgcct	23
GSTM1	NM_000561	S2026/GSTM1.r1	SEQ ID NO: 31	GGCCCAGCTTGAATTTTTCA	20
GSTM1	NM_000561	S2027/GSTM1.f1	SEQ ID NO: 32	AAGCTATGAGGAAAAGAAGTACACGAT	27
GSTM1	NM_000561	S4739/GSTM1.p1	SEQ ID NO: 33	TCAGCCACTGGCTTCTGTCATAATCAGGAG	30
GUS	NM_000181	S0139/GUS.f1	SEQ ID NO: 34	CCCACTCAGTAGCCAAGTCA	20
GUS	NM_000181	S0141/GUS.r1	SEQ ID NO: 35	CACGCAGGTGGTATCAGTCT	20
GUS	NM_000181	S4740/GUS.p1	SEQ ID NO: 36	TCAAGTAAACGGGCTGTTTTCCAAACA	27
HER2	NM_004448	S0142/HER2.f3	SEQ ID NO: 37	CGGTGTGAGAAGTGCAGCAA	20
HER2	NM_004448	S0144/HER2.r3	SEQ ID NO: 38	CCTCTCGCAAGTGCTCCAT	19
HER2	NM_004448	S4729/HER2.p3	SEQ ID NO: 39	CCAGACCATAGCACACTCGGGCAC	24
Ki-67	NM_002417	S0436/Ki-67.f2	SEQ ID NO: 40	CGGACTTTGGGTGCGACTT	19
Ki-67	NM_002417	S0437/Ki-67.r2	SEQ ID NO: 41	TTACAACTCTTCCACTGGGACGAT	24
Ki-67	NM_002417	S4741/Ki-67.p2	SEQ ID NO: 42	CCACTTGTCGAACCACCGCTCGT	23
MYBL2	NM_002466	S3270/MYBL2.f1	SEQ ID NO: 43	GCCGAGATCGCCAAGATG	18
MYBL2	NM_002466	S3271/MYBL2.r1	SEQ ID NO: 44	CTTTTGATGGTAGAGTTCCAGTGATTC	27
MYBL2	NM_002466	S4742/MYBL2.p1	SEQ ID NO: 45	CAGCATTGTCTGTCCTCCCTGGCA	24

PR	NM_000926	S1336/PR.f6	SEQ ID NO: 46	GCATCAGGCTGTCATTATGG	20
PR	NM_000926	S1337/PR.r6	SEQ ID NO: 47	AGTAGTTGTGCTGCCCTTCC	20
PR	NM_000926	S4743/PR.p6	SEQ ID NO: 48	TGTCCTTACCTGTGGGAGCTGTAAGGTC	28
RPLPO	NM_001002	S0256/RPLPO.f2	SEQ ID NO: 49	CCATTCTATCATCAACGGGTACAA	24
RPLPO	NM_001002	S0258/RPLPO.r2	SEQ ID NO: 50	TCAGCAAGTGGGAAGGTGTAATC	23
RPLPO	NM_001002	S4744/RPLPO.p2	SEQ ID NO: 51	TCTCCACAGACAAGGCCAGGACTCG	25
STK15	NM_003600	S0794/STK15.f2	SEQ ID NO: 52	CATCTTCCAGGAGGACCACT	20
STK15	NM_003600	S0795/STK15.r2	SEQ ID NO: 53	TCCGACCTTCAATCATTTCA	20
STK15	NM_003600	S4745/STK15.p2	SEQ ID NO: 54	CTCTGTGGCACCCTGGACTACCTG	24
STMY3	NM_005940	S2067/STMY3.f3	SEQ ID NO: 55	CCTGGAGGCTGCAACATACC	20
STMY3	NM_005940	S2068/STMY3.r3	SEQ ID NO: 56	TACAATGGCTTTGGAGGATAGCA	23
STMY3	NM_005940	S4746/STMY3.p3	SEQ ID NO: 57	ATCCTCCTGAAGCCCTTTTCGCAGC	25
SURV	NM_001168	S0259/SURV.f2	SEQ ID NO: 58	TGTTTTGATTCCCGGGCTTA	20
SURV	NM_001168	S0261/SURV.r2	SEQ ID NO: 59	CAAAGCTGTCAGCTCTAGCAAAAG	24
SURV	NM_001168	S4747/SURV.p2	SEQ ID NO: 60	TGCCTTCTTCCTCCCTCACTTCTCACCT	28
TFRC	NM_003234	S1352/TFRC.f3	SEQ ID NO: 61	GCCAACTGCTTTCATTTGTG	20
TFRC	NM_003234	S1353/TFRC.r3	SEQ ID NO: 62	ACTCAGGCCCATTTCCTTTA	20
TFRC	NM_003234	S4748/TFRC.p3	SEQ ID NO: 63	AGGGATCTGAACCAATACAGAGCAGACA	28

TABLE 2

Gene	Accession No.	Artificial Sequence PCR Amplicon	Amplicon Sequence
B-actin	NM_001101	SEQ ID NO: 64	CAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGCCCCTCCATCGTCCACCGCAAATGC
BAG1	NM_004323	SEQ ID NO: 65	CGTTGTCAGCACTTGGAATACAAGATGGTTGCCGGGTCATGTTAATTGGGAAAAAGAACAGTCCACAGGAAGAGGTTGAAC
Bcl2	NM_000633	SEQ ID NO: 66	CAGATGGACCTAGTACCCACTGAGATTTCCACGCCGAAGGACAGCGATGGGAAAAATGCCCTTAAATCATAGG
CCNB1	NM_031966	SEQ ID NO: 67	TTCAGGTTGTTGCAGGAGACCATGTACATGACTGTCTCCATTATTGATCGGTTCATGCAGAATAATTGTGTGCCCAAGAAGATG
CD68	NM_001251	SEQ ID NO: 68	TGGTTCCCAGCCCTGTGTCCACCTCCAAGCCCAGATTCAGATTCGAGTCATGTACACAACCCAGGGTGGAGGAG
CEGP1	NM_020974	SEQ ID NO: 69	TGACAATCAGCACACCTGCATTCACCGCTCGGAAGAGGGCCTGAGCTGCATGAATAAGGATCACGGCTGTAGTCACA
CTSL2	NM_001333	SEQ ID NO: 70	TGTCTCACTGAGCGAGCAGAATCTGGTGGACTGTTCGCGTCCTCAAGGCAATCAGGGCTGCAATGGT
EstR1	NM_000125	SEQ ID NO: 71	CGTGGTGCCCCTCTATGACCTGCTGCTGGAGATGCTGGACGCCCACCGCCTACATGCGCCCACTAGCC
GAPDH	NM_002046	SEQ ID NO: 72	ATTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGGGAAGCTTGTCATCAATGGAAATCCCATC
GRB7	NM_005310	SEQ ID NO: 73	CCATCTGCATCCATCTTGTTTGGGCTCCCCACCCTTGAGAAGTGCCTCAGATAATACCCTGGTGGCC
GSTM1	NM_000561	SEQ ID NO: 74	AAGCTATGAGGAAAAGAAGTACACGATGGGGGACGCTCCTGATTATGACAGAAGCCAGTGGCTGAATGAAAAATTCAAGCTGGGCC
GUS	NM_000181	SEQ ID NO: 75	CCCACTCAGTAGCCAAGTCACAATGTTTGGAAAACAGCCCGTTTACTTGAGCAAGACTGATACCACCTGCGTG
HER2	NM_004448	SEQ ID NO: 76	CGGTGTGAGAAGTGCAGCAAGCCCTGTGCCCGAGTGTGCTATGGTCTGGGCATGGAGCACTTGCGAGAGG
Ki-67	NM_002417	SEQ ID NO: 77	CGGACTTTGGGTGCGACTTGACGAGCGGTGGTTCGACAAGTGGCCTTGCGGGCCGGATCGTCCCAGTGGAAGAGTTGTAA
MYBL2	NM_002466	SEQ ID NO: 78	GCCGAGATCGCCAAGATGTTGCCAGGGAGGACAGACAATGCTGTGAAGAATCACTGGAACTCTACCATCAAAAG
PR	NM_000926	SEQ ID NO: 79	GCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCACAACTACT
RPLPO	NM_001002	SEQ ID NO: 80	CCATTCTATCATCAACGGGTACAAACGAGTCCTGGCCTTGTCTGTGGAGACGGATTACACCTTCCCACTTGCTGA
STK15	NM_003600	SEQ ID NO: 81	CATCTTCCAGGAGGACCACTCTCTGTGGCACCCTGGACTACCTGCCCCCTGAAATGATTGAAGGTCGGA
STMY3	NM_005940	SEQ ID NO: 82	CCTGGAGGCTGCAACATACCTCAATCCTGTCCCAGGCCGGATCCTCCTGAAGCCCTTTTCGCAGCACTGCTATCCTCCAAAGCCATTGTA
SURV	NM_001168	SEQ ID NO: 83	TGTTTTGATTCCCGGGCTTACCAGGTGAGAAGTGAGGGAGGAAGAAGGCAGTGTCCCTTTTGCTAGAGCTGACAGCTTTG
TFRC	NM_003234	SEQ ID NO: 84	GCCAACTGCTTTCATTTGTGAGGGATCTGAACCAATACAGAGCAGACATAAAGGAAATGGGCCTGAGT

2004258085 28 Apr 2010

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for determining the likelihood of breast cancer recurrence or response to hormonal therapy in a mammalian subject comprising:

(a) measuring the expression levels of the RNA transcripts of at least four of the following genes GRB7, HER2, EstR1(ER), PR, Bcl2, CEGP1, SURV, Ki.67, MYBL2, CCNB1, STK15, CTSL2, and STMY3 according to (b), or their expression products in a biological sample comprising a breast tumor cell obtained from said subject;

(b) creating the following gene subsets each comprising an expression level of at least one of said genes or a substitute gene which coexpresses with each of the selected genes with a Pearson correlation coefficient of ≥ 0.40:

(i) a growth factor subset: GRB7 and HER2;

(ii) an estrogen receptor subset: EstR1(ER), PR, Bcl2 and CEGP1;

(iii) a proliferation subset: SURV, Ki.67, MYBL2, CCNB1 and STK15; and

(iv) an invasion subset: CTSL2 and STMY3;

(c) calculating the recurrence score (RS) for said subject by weighting the measured expression levels of each of the gene subsets by contribution to breast cancer recurrence;

(d) using said RS to determine the likelihood of breast cancer recurrence or response to therapy; and

(e) creating a report summarizing the result of said determination.

2. The method of claim 1 further comprising determining the expression levels of RNA transcripts of CD68, GSTM1 and BAG1 or their expression products, or corresponding substitute genes or their expression products, and including the contribution of the measured expression levels of the genes or substitute genes to breast cancer recurrence in calculating the RS,

wherein a higher RS represents an increased likelihood of breast cancer recurrence, and

2004258085 28 Apr 2010

wherein a substitute gene for CD68 is selected from the group consisting of CTSB, CD18, CTSL, HLA.DPB1, and MMP9, a substitute gene for GSTM1 is selected from the group consisting of GSTM2, GSTM3, GSTM4, GSTM5, MYH11, GSN, and ID1, and a substitute gene for BAG1 is selected from the group consisting of Bcl2, GATA3, DHPS, and HNF3A.

3. The method of claim 1 or claim 2 wherein the expression level of said genes is measured by determining protein expression levels.

4. The method of any one of claims 1 to 3 wherein said subject is human.

5. The method of any one of claims 1 to 4 wherein the cancer is lymph node negative breast cancer.

6. The method of any one of claims 1 to 5 wherein the individual contribution of each gene of gene subsets (i) - (iv) is weighted separately.

7. The method of claim 6 comprising multiplying in gene subset (i) the expression levels of the RNA transcript of each of GRB7 and HER2, or their expression products, by a factor between 0 and 1, representing their individual contributions to breast cancer recurrence.

8. The method of claim 6 comprising weighting equally the individual contributions of the genes listed in gene subset (ii), or their expression products, to breast cancer recurrence.

9. The method of claim 6 comprising weighting equally the individual contributions of the genes listed in gene subset (iii), or their expression products, to breast cancer recurrence.

10. The method of claim 6 comprising weighting equally the individual contributions of each gene listed in gene subset (iv), or their expression products, to breast cancer recurrence.

11. The method of any one of claims 1 to 10 wherein increased levels of the RNA transcripts of the genes in gene subsets (i), (iii) and (iv), or their expression products, are associated with increased risk of breast cancer recurrence, and are assigned a positive value.

12. The method of any one of claims 1 to 10 wherein increased levels of the RNA transcripts of the genes in gene subset (ii), or their expression products, are associated with decreased risk of breast cancer recurrence, and are assigned a negative value.

13. The method of any one of claims 2 to 12 wherein increased level of the RNA transcript of CD68, or its expression product, is associated with increased risk of breast cancer recurrence, and is assigned a positive value.

14. The method of any one of claims 2 to 12 wherein increased levels of the RNA transcripts of GSTM1 and BAG1, or their expression products, are associated with decreased risk of breast cancer recurrence, and are assigned a negative value.

15. The method of any one of claims 1 to 14 wherein said biological sample is selected from the group consisting of fresh tumor tissue, fine needle aspirates, peritoneal fluid, ductal lavage and pleural fluid.

16. The method of any one of claims 1 to 14 wherein said biological sample is a tumor sample.

17. The method of claim 16 wherein said tumor sample is obtained from a fixed, paraffin embedded tumor tissue.

2004258085 28 Apr 2010

18. The method of claim 17 wherein said tissue has been obtained by biopsy.

19. The method of claim 17 or claim 18 wherein the expression levels of said RNA transcripts are determined by RT-PCR.

20. The method of any one of claims 17 to 19 wherein said RNA is fragmented.

21. The method of any one of claims 1 to 20 further comprising the step of analyzing the RS using Kaplan-Meier survival curves.

22. The method of any one of claims 1 to 21 wherein the contributions of gene subsets (i) and (iii) are included at a threshold value until the contributions exceed the threshold value at which point the contributions are included as a total expression level of the RNA transcripts of the genes in said subsets, or their expression products.

23. A method for determining the likelihood of breast cancer recurrence or response to hormonal therapy in a mammalian subject comprising:

(a) measuring expression levels of RNA transcripts for at least four of a plurality of genes or their expression products, in a biological sample containing tumor cells obtained from said subject, wherein the plurality of genes are grouped in gene subsets comprising:

(i) a growth factor subset;

(ii) an estrogen receptor subset;

(iii) a proliferation subset; and

(iv) an invasion subset,

wherein at least one gene within subset (i) is selected from the group consisting of: GFB7; HER2; Q9BRT3; TCAP; PNMT; ML64; IPPD; Q9H7G1; Q9HBS1; Q9Y220; PSMD3; and CSF3,

wherein at least one gene within subset (ii) is selected from the group consisting of: EstR1(ER); PR; Bcl2; CEGP1; HNF3A; ErbB3; GATA3; BECN1; IGF1R; AKT2; DHPS; BAG1; hENT1; TOP2B; MDM2; CCND1; DKFZp586M0723; NPD009;

2004258085 28 Apr 2010

B.Catenin; IRS1; Bclx; RBM5; PTEN; A.Catenin; KRT18; ZNF217; ITGA7; GSN; MTA1; G.Catenin; DR5; RAD51C; BAD; TP53BP1; RIZ1; IGFBP2; RUNX1; PPM1D; TFF3; S100A8; P28; SFRS5; and IGFBP2,

wherein at least one gene within subset (iii) is selected from the group consisting of: SURV; Ki67; MYBL2; CCNB1; STK15; C20.orfl, TOP2A; CDC20; KNSL2; MELK; TK1; NEK2; LMNB1; PTTG1; BUB1; CCNE2; FLJ20354; MCM2; RAD54L; PRO2000; PCNA; Chk1; NME1; TS; FOXM1; AD024; and HNRPAB, and

wherein at least one gene within subset (iv) is selected from the group consisting of: CTSL2 and STMY3;

(b) calculating a recurrence score (RS) for said subject by weighting the measured expression levels for each of the gene subsets by contribution to breast cancer recurrence;

(c) using the RS to determine a likelihood of breast cancer recurrence or response to therapy; and

(d) creating a report based on the likelihood of breast cancer recurrence or response to therapy.

24. The method of claim 23 further comprising measuring expression levels of RNA transcripts of CD68, GSTM1 and BAG1, or a substitute gene thereof, or their expression products, and including the contribution of measured expression levels of CD68, GSTM1 and BAG1, or substitute genes, to breast cancer recurrence in calculating the RS,

wherein a higher RS represents an increased likelihood of breast cancer recurrence,

wherein a substitute gene for CD68 is selected from the group consisting of: CTSB; CD18; CTSL; HLA.DPB1; and MMP9,

wherein a substitute gene for GSTM1 is selected from the group consisting of: GSTM2; GSTM3; GSTM4; GSTM5; MYH11; GSN; and ID1, and

wherein a substitute gene for BAG1 is selected from the group consisting of: Bcl2, GATA3, DHPS, and HNF3A.

25. The method of claim 24 wherein an increased level of the RNA transcript of CD68, or a substitute gene thereof, or its expression product, is associated with an increased risk of breast cancer recurrence, and is assigned a positive value.

26. The method of claim 24 wherein increased levels of the RNA transcripts of GSTM1 and BAG1, or a substitute gene thereof, or their expression products, are associated with a decreased risk of breast cancer recurrence, and are assigned a negative value.

27. The method of any one of claims 23 to 26 wherein said subject is human.

28. The method of any one of claims 23 to 27 wherein the cancer is lymph node negative breast cancer.

29. The method of any one of claims 23 to 28 wherein the contributions of subsets (i) and (iii) are included at a threshold value until the contributions exceed the threshold value, at which point the contributions are included as a total expression level of the RNA transcripts of the genes, or substitute genes, in said subsets, or their expression products.

30. The method of any one of claims 23 to 29 wherein within each of gene subsets (i) - (iv), individual contributions of each gene or substitute gene included is weighted separately.

31. The method of any one of claims 23 to 30 comprising multiplying expression levels of the genes or substitute genes of gene subset (i), or their expression products, by a factor between 0 and 1, representing their individual contributions to breast cancer recurrence.

32. The method of claim 31 comprising weighting equally individual contributions of the genes or substitute genes of gene subset (ii), or their expression products, to breast cancer recurrence.

2004258085 28 Apr 2010

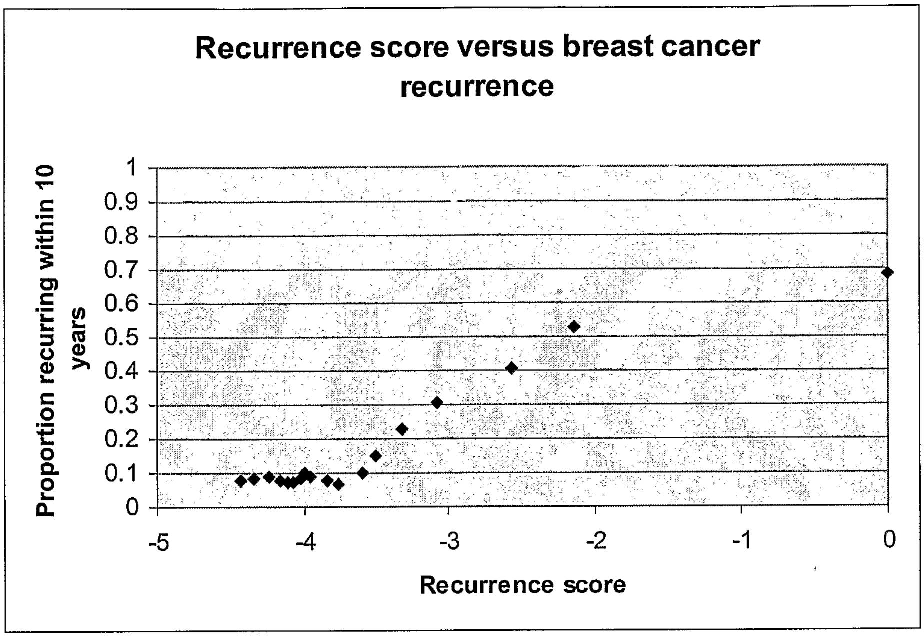
33. The method of claim 31 comprising weighting equally individual contributions of the genes or substitute genes of gene subset (iii), or their expression products, to breast cancer recurrence.

34. The method of claim 31 comprising weighting equally individual contributions of each gene or substitute genes of gene subset (iv), or their expression products, to breast cancer recurrence.

35. The method of any one of claims 23 to 34 wherein increased levels of RNA transcripts of the genes or substitute genes of gene subsets (i), (iii) and (iv), or their expression products, are associated with increased risk of breast cancer recurrence, and are assigned a positive value.

36. The method of any one of claims 23 to 34 wherein increased levels of RNA transcripts of the genes or substitute genes of gene subset (ii), or their expression products, are associated with decreased risk of breast cancer recurrence, and are assigned a negative value.

37. A method according to claim 1 or claim 23, substantially as hereinbefore described with reference to any one of the examples or figures.


Recurrence score versus breast cancer recurrence
Proportion recurring within 10 years
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0.9
0.8
0.7
0.6
0.5
0.4
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0.2
0.1
0
-5
-4
-3
-2
-1
0
Recurrence score

FIGURE 1A

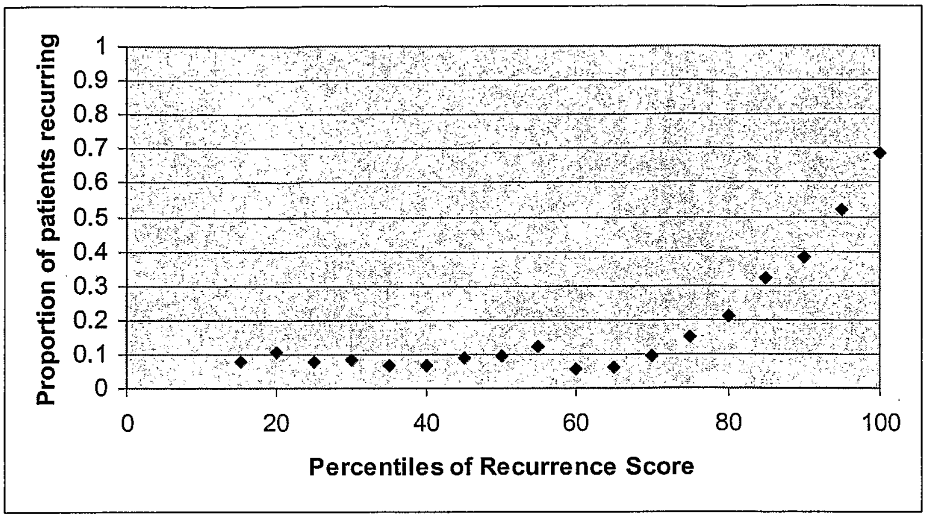
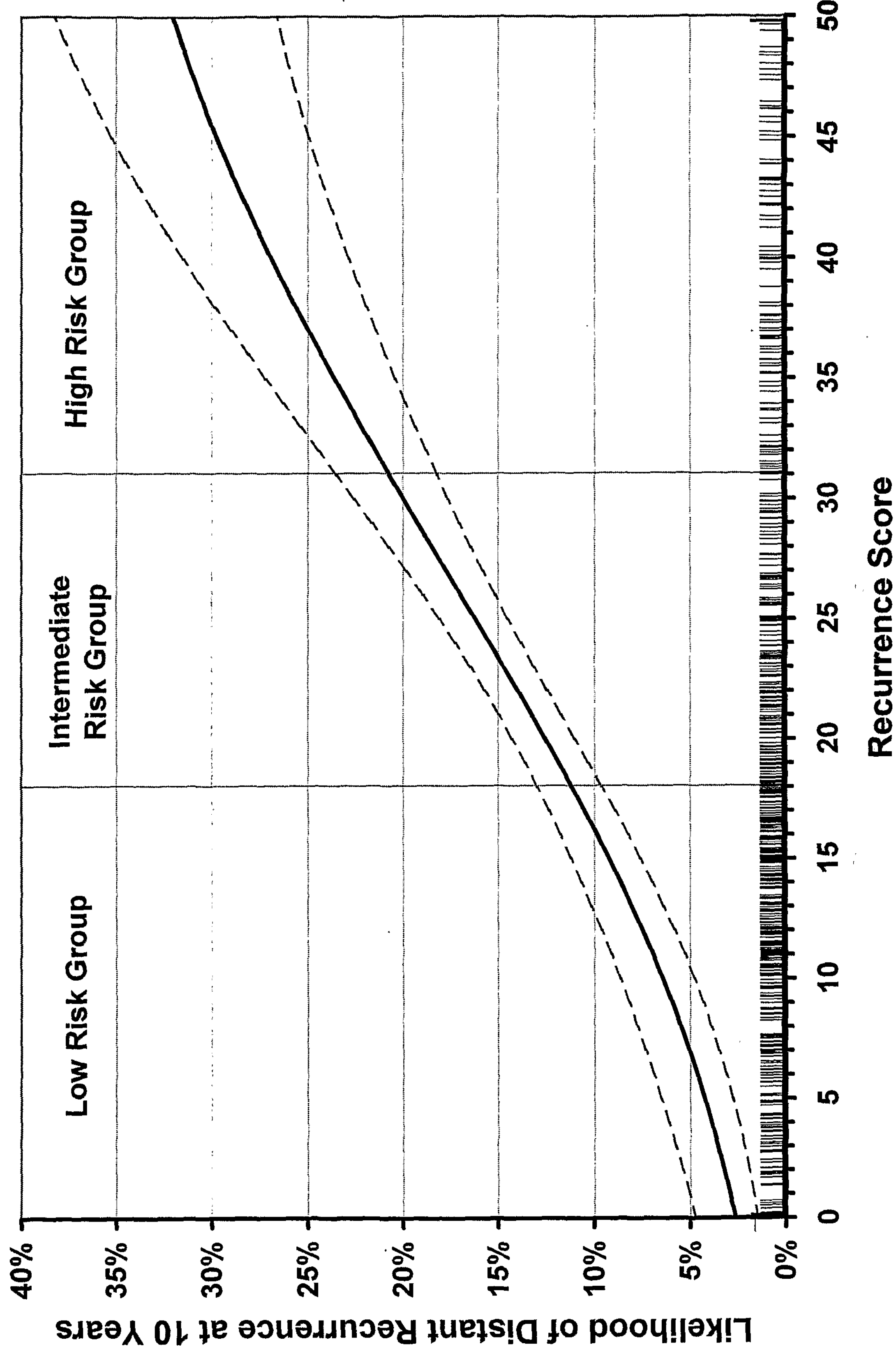

Proportion of patients recurring
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
Percentiles of Recurrence Score

FIGURE 1B

FIGURE 2



Low Risk Group
Intermediate Risk Group
High Risk Group
Recurrence Score
0
5
10
15
20
25
30
35
40
45
50
Likelihood of Distant Recurrence at 10 Years
0%
5%
10%
15%
20%
25%
30%
35%
40%

SEQUENCE LISTING

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BRYANT, John L.
PAIK, Soonmyung
SHAK, Steven

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