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DESCRIPTION

Background of the Invention

Field of the Invention

[0001] The present invention provides genes and gene sets the expression of which is important in the diagnosis and/or prognosis of breast cancer.

Description of the Related Art

[0002] Oncologists have a number of treatment options available to them, including different combinations of chemotherapeutic drugs that are characterized as "standard of care," and a number of drugs that do not carry a label claim for particular cancer, but for which there is evidence of efficacy in that cancer. Best likelihood of good treatment outcome requires that patients be assigned to optimal available cancer treatment, and that this assignment be made as quickly as possible following diagnosis.

[0003] Currently, diagnostic tests used in clinical practice are single analyte, and therefore do not capture the potential value of knowing relationships between dozens of different 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 (DNA or RNA) from small amounts of material. Thus, traditionally fixed tissue has been rarely used other than for immunohistochemistry detection of proteins.

[0004] Recently, 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); Bhattacharjee 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); Sorlie et al., Proc. Natl. Acad. Sci. USA 98:10869-10874 (2001); Yan et al., Cancer Res. 61:8375-8380 (2001)). However, these studies mostly focus on improving and refining the already established classification of various types of cancer, including breast cancer, and generally do not provide new insights into the relationships of the differentially expressed genes, and do not link the findings to treatment strategies in order to improve the clinical outcome of cancer therapy.

[0005] Although modern molecular biology and biochemistry have revealed hundreds of genes whose activities influence the behavior of tumor cells, state of their differentiation, 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 to 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 with the Her2 antagonist drug Herceptin® (Genentech, Inc., South San Francisco, CA).

[0006] Despite recent advances, the challenge of cancer treatment remains to target specific treatment regimens to pathogenically distinct tumor types, and ultimately personalize tumor treatment in order to maximize outcome. Hence, a need exists for tests that simultaneously provide predictive information about patient responses to the variety of treatment options. This is particularly true for breast cancer, the biology of which is poorly understood. 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 transcriptional factors (Perou et al., Nature 406:747-752 (2000)) does not reflect the cellular and molecular heterogeneity of breast cancer, and does not allow the design of treatment strategies maximizing patient response.

[0007] WO02/10436 discloses a method for prognostic classification of breast cancer based on determining the expression level of one or more RNA transcripts selected from a list of 57 genes. These 57 genes are found to be preferentially expressed in tumors of poor prognosis vs. tumors of good prognosis. The long-term survival of patients without recurrence is measured through the mitotic activity index, which is an indicator of death, recurrence and metastases in breast cancer.

[0008] RASCHELLA GIUSEPPE ET AL ("Expression of B-myb in neuroblastoma tumors is a poor prognostic factor independent from MYCN amplification" CANCER RESEARCH, vol. 59, no. 14, pages 3365-3368) disclose MYBL2 as a marker of poor prognosis in neuroblastoma.

Summary of the Invention

[0009] The present disclosure provides a set of genes, the expression of which has prognostic value, specifically with respect to disease-free survival.

[0010] The present invention accommodates the use of archived paraffin-embedded biopsy material for assay of all markers in the set, and therefore is compatible with the most widely available type of biopsy material. It is also compatible with several different methods of tumour tissue harvest, for example, via core biopsy or fine needle aspiration. Further, for each member of the gene set, the invention specifies oligonucleotide sequences that can be used in the test.

[0011] According to an aspect of the present invention, there is provided a method of predicting the likelihood of long-term survival of a breast cancer patient without recurrence of breast cancer as specified in claim 1.

[0012] In a particular embodiment, the breast cancer is invasive breast carcinoma.

[0013] In a further embodiment, RNA is isolated from a fixed, wax-embedded breast cancer tissue specimen of the patient. Isolation may be performed by a technique known in the art, for example from core biopsy tissue or fine needle aspirate cells.

[0014] The patient may be diagnosed with estrogen receptor (ER)-positive invasive breast cancer.

[0015] The method may include creating a report summarizing the data obtained by the gene expression analysis.

[0016] The report may, for example, include prediction of the likelihood of long term survival of the patient and/or recommendation for a treatment modality of said patient.

Brief Description of the Drawings

[0017]

Table 1 is a list of genes, expression of which correlate with breast cancer survival. Results from a retrospective clinical trial. Binary statistical analysis.

Table 2 is a list of genes, expression of which correlates with breast cancer survival in estrogen receptor (ER) positive patients. Results from a retrospective clinical trial. Binary statistical

analysis.

Table 3 is a list of genes, expression of which correlates with breast cancer survival in estrogen receptor (ER) negative patients. Results from a retrospective clinical trial. Binary statistical analysis.

Table 4 is a list of genes, expression of which correlates with breast cancer survival. Results from a retrospective clinical trial. Cox proportional hazards statistical analysis.

Tables 5A and B show a list of genes, expression of which correlate with breast cancer survival. Results from a retrospective clinical trial. The table includes accession numbers for the genes, and amplicon sequences used for PCR amplification.

Tables 6A-6F The table includes sequences for the forward and reverse primers (designated by "f" and "r", respectively) and probes (designated by "p") used for PCR amplification of the amplicons listed in Tables 5A-B.

Detailed Description of the Preferred Embodiment

A. Definitions

[0018] 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.

[0019] 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.

[0020] The term "microarray" refers to an ordered arrangement of hybridizable array elements, preferably polynucleotide probes, on a substrate.

[0021] The term "polynucleotide," when used in singular or plural, generally refers to any polyribonucleotide or polydeoxynucleotide, 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.

[0022] 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.

[0023] 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.

[0024] 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 the proportion of the number of copies made of the particular gene expressed.

[0025] The term "diagnosis" is used herein to refer to the identification of a molecular or pathological state, disease or condition, such as the identification of a molecular subtype of head and neck cancer, colon cancer, or other type of cancer.

[0026] 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.

[0027] 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 of the primary tumor and/or chemotherapy for a certain period of time without cancer recurrence. The predictive methods of the present invention can be used clinically to make treatment decisions by choosing the most appropriate treatment modalities for any particular patient. The predictive methods of the present invention 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.

[0028] The term "long-term" survival is used herein to refer to survival for at least 3 years, more preferably for at least 8 years, most preferably for at least 10 years following surgery or other treatment.

[0029] 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.

[0030] 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.

[0031] The "pathology" of 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.

[0032] "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).

[0033] "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.

[0034] "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 than 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.

[0035] 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.

[0036] The terms "expression threshold," and "defined expression threshold" are used interchangeably and refer to the level of a gene or gene product in question above which the gene or gene product serves as a predictive marker for patient survival without cancer recurrence. The threshold is defined experimentally from clinical studies such as those described in the Example below. The expression threshold can be selected either for maximum sensitivity, or for maximum selectivity, or for minimum error. The determination of the expression threshold for any situation is well within the knowledge of those skilled in the art.

B. Detailed Description

[0037] 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).

1. Gene Expression Profiling

[0038] In general, methods of gene expression profiling 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).

2. Reverse Transcriptase PCR (RT-PCR)

[0039] Of the techniques listed above, the most sensitive and most flexible quantitative method is RT-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.

[0040] 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.

[0041] 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.

[0042] 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 avian 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.

[0043] 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 labelled 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.

[0044] TaqMan® RT-PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700™ Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, 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 7700™ Sequence Detection System™. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optics cables for all 96 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

[0045] 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 (C_t).

[0046] 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.

[0047] A more recent variation of the RT-PCR technique is the real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic 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).

[0048] The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: T.E. Godfrey et al., J. Molec. Diagnostics 2: 84-91 [2000]; K. Specht et al., Am. J. Pathol. 158: 419-29 [2001]). Briefly, a representative process starts with cutting about 10 μ m thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by RT-PCR.

[0049] According to one aspect of the present invention, PCR primers and probes are designed based upon intron sequences present in the gene to be amplified. In this embodiment, the first step in the primer/probe design is the delineation of intron sequences within the genes. This can be done by publicly available software, such as the DNA BLAT software developed by Kent, W.J., Genome Res. 12(4):656-64 (2002), or by the BLAST software including its variations. Subsequent steps follow well established methods of PCR primer and probe design.

[0050] In order to avoid non-specific signals, it is important to mask repetitive sequences within the introns when designing the primers and probes. This can be easily accomplished by using the Repeat Masker program available on-line through the Baylor College of Medicine, which screens DNA sequences against a library of repetitive elements and returns a query sequence in which the repetitive elements are masked. The masked intron sequences can then be used to design primer and probe sequences using any commercially or otherwise publicly available primer/probe design packages, such as Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Steve Rozen and Helen J. Skalaetsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386).

[0051] The most important factors considered in PCR primer design include primer length, melting temperature (T_m), and G/C content, specificity, complementary primer sequences, and 3'-end sequence. In general, optimal PCR primers are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. T_m's between 50 and 80 °C, e.g. about 50 to 70 °C are typically preferred.

[0052] For further guidelines for PCR primer and probe design see, e.g. Dieffenbach, C.W. et al., "General Concepts for PCR Primer Design" in: PCR Primer, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1995, pp. 133-155; Innis and Gelfand, "Optimization of PCRs" in: PCR Protocols, A Guide to Methods and Applications, CRC Press, London, 1994, pp. 5-11; and Plasterer, T.N. Primerselect: Primer and probe design. Methods Mol. Biol. 70:520-527 (1997).

3. Microarrays

[0053] 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.

[0054] 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.

[0055] 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)

[0056] 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. MassARRAY Technology

[0057] The MassARRAY (Sequenom, San Diego, California) technology is an automated, high-throughput method of gene expression analysis using mass spectrometry (MS) for detection. According to this method, following the isolation of RNA, reverse transcription and PCR amplification, the cDNAs are subjected to primer extension. The cDNA-derived primer

extension products are purified, and dispensed on a chip array that is pre-loaded with the components needed for MALDI-TOF MS sample preparation. The various cDNAs present in the reaction are quantitated by analyzing the peak areas in the mass spectrum obtained.

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

[0058] 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 3×10^6 microbeads/cm²). 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.

7. Immunohistochemistry

[0059] Immunohistochemistry methods are also suitable for detecting the expression levels of prognostic markers. 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.

8. Proteomics

[0060] 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. by mass 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 products of prognostic markers.

9. General Description of the mRNA Isolation, Purification and Amplification

[0061] The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: T.E. Godfrey et al. J. Molec. Diagnostics 2: 84-91 [2000]; K. Specht et al., Am. J. Pathol. 158: 419-29 [2001]). Briefly, a representative process starts with cutting about 10 µm thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by RT-PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumor sample examined.

10. Breast Cancer Gene Set, Assayed Gene Subsequences, and Clinical Application of Gene Expression Data

[0062] An important aspect of the present invention is to use the measured expression of certain genes by 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 known housekeeping genes, such as GAPDH and Cyp1. 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). On a gene-by-gene basis, measured normalized amount of a patient tumor mRNA is compared to the amount found in a breast cancer tissue reference set. The number (N) of breast cancer tissues in this reference set should be sufficiently high to ensure that different reference sets (as a whole) behave essentially the same way. If this condition is met, the identity of the individual breast cancer tissues present in a particular set will have no significant impact on the relative amounts of the genes assayed. Usually, the breast cancer tissue reference set consists of at least about 30, preferably at least about 40 different FPE breast cancer tissue specimens. Unless noted otherwise, normalized expression levels for each mRNA/tested tumor/patient will be expressed as a percentage of the expression level measured in the reference set. More specifically, the reference set of a sufficiently high number (e.g. 40) of tumors yields a distribution of normalized levels of each mRNA species. The level measured in a particular tumor sample to be analyzed falls at some percentile within this range, which can be determined by methods well known in the art. Below, unless noted otherwise, reference to expression levels of a gene assume normalized expression relative to the reference set although this is not always explicitly stated.

[0063] Further details of the invention will be described in the following non-limiting Example

Example

A Phase II Study of Gene Expression in 79 Malignant Breast Tumors

[0064] A gene expression study was designed and conducted with the primary goal to molecularly characterize gene expression in paraffin-embedded, fixed tissue samples of invasive breast ductal carcinoma, and to explore the correlation between such molecular profiles and disease-free survival.

Study design

[0065] Molecular assays were performed on paraffin-embedded, formalin-fixed primary breast tumor tissues obtained from 79 individual patients diagnosed with invasive breast cancer. All patients in the study had 10 or more positive nodes. Mean age was 57 years, and mean clinical tumor size was 4.4 cm. 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

[0066] Each representative tumor block was characterized by standard histopathology for diagnosis, semi-quantitative assessment of amount of tumor, and tumor grade. A total of 6 sections (10 microns in thickness each) were prepared and placed in two Costar Brand Microcentrifuge Tubes (Polypropylene, 1.7 mL tubes, clear; 3 sections in each tube). If the tumor constituted less than 30% of the total specimen area, the sample may have been crudely dissected by the pathologist, using gross microdissection, putting the tumor tissue directly into the Costar tube.

[0067] 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

[0068] mRNA was extracted and purified from fixed, paraffin-embedded tissue samples, and prepared for gene expression analysis as described in section 9 above.

[0069] 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. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optics cables for all 384 wells, and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

Analysis and Results

[0070] Tumor tissue was analyzed for 185 cancer-related genes and 7 reference genes. The threshold cycle (CT) values for each patient were normalized based on the median of the 7 reference genes for that particular patient. Clinical outcome data were available for all patients from a review of registry data and selected patient charts.

[0071] Outcomes were classified as:

0 died due to breast cancer or to unknown cause or alive with breast cancer recurrence;

1 alive without breast cancer recurrence or died due to a cause other than breast cancer

[0072] Analysis was performed by:

1. Analysis of the relationship between normalized gene expression and the binary outcomes of 0 or 1.
2. Analysis of the relationship between normalized gene expression and the time to outcome (0 or 1 as defined above) where patients who were alive without breast cancer recurrence or who died due to a cause other than breast cancer were censored. This approach was used to evaluate the prognostic impact of individual genes and also sets of multiple genes.

Analysis of patients with invasive breast carcinoma by binary approach

[0073] In the first (binary) approach, analysis was performed on all 79 patients with invasive breast carcinoma. A t test was performed on the groups of patients classified as either no recurrence and no breast cancer related death at three years, versus recurrence, or breast cancer-related death at three years, and the p-values for the differences between the groups for each gene were calculated.

[0074] Table 1 lists the 47 genes for which the p-value for the differences between the groups was <0.10. The first column of mean expression values pertains to patients who neither had a metastatic recurrence of nor died from breast cancer. The second column of mean expression values pertains to patients who either had a metastatic recurrence of or died from breast cancer.

Table 1

	Mean	Mean	t-value	df	p	Valid N	Valid N
Bcl2	-0.15748	-1.22816	4.00034	75	0.000147	35	42
PR	-2.67225	-5.49747	3.61540	75	0.000541	35	42
IGF1R	-0.59390	-1.71506	3.49158	75	0.000808	35	42
BAG1	0.18844	-0.68509	3.42973	75	0.000985	35	42
CD68	-0.52275	0.10983	-3.41186	75	0.001043	35	42
EstR1	-0.35581	-3.00699	3.32190	75	0.001384	35	42
CTSL	-0.64894	-0.09204	-3.26781	75	0.001637	35	42
IGFBP2	-0.81181	-1.78398	3.24158	75	0.001774	35	42
GATA3	1.80525	0.57428	3.15608	75	0.002303	35	42
TP53BP2	-4.71118	-6.09289	3.02888	75	0.003365	35	42
EstR1	3.67801	1.64693	3.01073	75	0.003550	35	42
CEGP1	-2.02566	-4.25537	2.85620	75	0.005544	35	42
SURV	-3.67493	-2.96982	-2.70544	75	0.008439	35	42
p27	0.80789	0.28807	2.55401	75	0.012678	35	42
Chk1	-3.37981	-2.80389	-2.46979	75	0.015793	35	42
BBC3	-4.71789	-5.62957	2.46019	75	0.016189	35	42
ZNF217	1.10038	0.62730	2.42282	75	0.017814	35	42
EGFR	-2.88172	-2.20556	-2.34774	75	0.021527	35	42
CD9	1.29955	0.91025	2.31439	75	0.023386	35	42
MYBL2	-3.77489	-3.02193	-2.29042	75	0.024809	35	42
HIF1A	-0.44248	0.03740	-2.25950	75	0.026757	35	42
GRB7	-1.96063	-1.05007	-2.25801	75	0.026854	35	42
pS2	-1.00691	-3.13749	2.24070	75	0.028006	35	42
RIZ1	-7.62149	-8.38750	2.20226	75	0.030720	35	42
ErbB3	-6.89508	-7.44326	2.16127	75	0.033866	35	42
TOP2B	0.45122	0.12665	2.14616	75	0.035095	35	42
MDM2	1.09049	0.69001	2.10967	75	0.038223	35	42

	Mean	Mean	t-value	df	p	Valid N	Valid N
PRAME	-6.40074	-7.70424	2.08126	75	0.040823	35	42
GUS	-1.51683	-1.89280	2.05200	75	0.043661	35	42
RAD51C	-5.85618	-6.71334	2.04575	75	0.044288	35	42
AIB1	-3.08217	-2.28784	-2.00600	75	0.048462	35	42
STK15	-3.11307	-2.59454	-2.00321	75	0.048768	35	42
GAPDH	-0.35829	-0.02292	-1.94326	75	0.055737	35	42
FHIT	-3.00431	-3.67175	1.86927	75	0.065489	35	42
KRT19	2.52397	2.01694	1.85741	75	0.067179	35	42
TS	-2.83607	-2.29048	-1.83712	75	0.070153	35	42
GSTM1	-3.69140	4.38623	1.83397	75	0.070625	35	42
G-Catenin	0.31875	-0.15524	1.80823	75	0.074580	35	42
AKT2	0.78858	0.46703	1.79276	75	0.077043	35	42
CCNB1	-4.26197	-3.51628	-1.78803	75	0.077810	35	42
PI3KC2A	-2.27401	-2.70265	1.76748	75	0.081215	35	42
FBXO5	-4.72107	-4.24411	-1.75935	75	0.082596	35	42
DR5	-5.80850	-6.55501	1.74345	75	0.085353	35	42
CIAP1	-2.81825	-3.09921	1.72480	75	0.088683	35	42
MCM2	-2.87541	-2.50683	-1.72061	75	0.089445	35	42
CCND1	1.30995	0.80905	1.68794	75	0.095578	35	42
EIF4E	-5.37657	-6.47156	1.68169	75	0.096788	35	42

[0075] In the foregoing Table 1, negative t-values indicate higher expression, associated with worse outcomes, and, inversely, higher (positive) t-values indicate higher expression associated with better outcomes. Thus, for example, elevated expression of the CD68 gene (t-value = -3.41, CT mean alive < CT mean deceased) indicates a reduced likelihood of disease free survival. Similarly, elevated expression of the BCL2 gene (t-value = 4.00; CT mean alive > CT mean deceased) indicates an increased likelihood of disease free survival.

[0076] Based on the data set forth in Table 1, the expression of any of the following genes in breast cancer above a defined expression threshold indicates a reduced likelihood of survival without cancer recurrence following surgery: Grb7, CD68, CTSL, Chk1, Her2, STK15, AIB1, SURV, EGFR, MYBL2, HIF1 α .

[0077] Based on the data set forth in Table 1, the expression of any of the following genes in breast cancer above a defined expression threshold indicates a better prognosis for survival without cancer recurrence following surgery: TP53BP2, PR, Bcl2, KRT14, EstR1, IGFBP2, BAG1, CEGP1, KLK10, β Catenin, GSTM1, FHIT, RIZ1, IGF1, BBC3, IGFR1, TBP, p27, IRS1, IGF1R, GATA3, CEGP1, ZNF217, CD9, pS2, ErbB3, TOP2B, MDM2, RAD51, and KRT19.

Analysis of ER positive patients by binary approach

[0078] 57 patients with normalized CT for estrogen receptor (ER) >0 (i.e., ER positive patients) were subjected to separate analysis. A t test was performed on the two groups of patients classified as either no recurrence and no breast cancer related death at three years, or recurrence or breast cancer-related death at three years, and the p-values for the differences between the groups for each gene were calculated. Table 2, below, lists the genes where the p-value for the differences between the groups was <0.105. The first column of mean expression values pertains to patients who neither had a metastatic recurrence nor died from breast cancer. The second column of mean expression values pertains to patients who either had a metastatic recurrence or died from breast cancer.

Table 2

	Mean	Mean	t-value	df	p	Valid N	Valid N
IGF1R	-0.13975	-1.00435	3.65063	55	0.000584	30	27
Bcl2	0.15345	-0.70480	3.55488	55	0.000786	30	27
CD68	-0.54779	0.19427	-3.41818	55	0.001193	30	27
HNF3A	0.39617	-0.63802	3.20750	55	0.002233	30	27
CTSL	-0.66726	0.00354	-3.20692	55	0.002237	30	27
TP53BP2	-4.81858	-6.44425	3.13698	55	0.002741	30	27
GATA3	2.33386	1.40803	3.02958	55	0.003727	30	27
BBC3	-4.54979	-5.72333	2.91943	55	0.005074	30	27
RAD51C	-5.63363	-6.94841	2.85475	55	0.006063	30	27
BAG1	0.31087	-0.50669	2.61524	55	0.011485	30	27
IGFBP2	-0.49300	-1.30983	2.59121	55	0.012222	30	27
FBXO5	-4.86333	-4.05564	-2.56325	55	0.013135	30	27
EstR1	0.68368	-0.66555	2.56090	55	0.013214	30	27
PR	-1.89094	-3.86602	2.52803	55	0.014372	30	27
SURV	-3.87857	-3.10970	-2.49622	55	0.015579	30	27
CD9	1.41691	0.91725	2.43043	55	0.018370	30	27
RB1	-2.51662	-2.97419	2.41221	55	0.019219	30	27
EPHX1	-3.91703	-5.85097	2.29491	55	0.025578	30	27
CEGP1	-1.18600	-2.95139	2.26608	55	0.027403	30	27
CCNB1	-4.44522	-3.35763	-2.25148	55	0.028370	30	27
TRAIL	0.34893	-0.56574	2.20372	55	0.031749	30	27
EstR1	4.60346	3.60340	2.20223	55	0.031860	30	27
DR5	-5.71827	-6.79088	2.14548	55	0.036345	30	27
MCM2	-2.96800	-2.48458	-2.10518	55	0.039857	30	27
Chk1	-3.46968	-2.85708	-2.08597	55	0.041633	30	27

	Mean	Mean	t-value	df	p	Valid N	Valid N
p27	0.94714	0.49656	2.04313	55	0.045843	30	27
MYBL2	-3.97810	-3.14837	-2.02921	55	0.047288	30	27
GUS	-1.42486	-1.82900	1.99758	55	0.050718	30	27
P53	-1.08810	-1.47193	1.92087	55	0.059938	30	27
HIF1A	-0.40925	0.11688	-1.91278	55	0.060989	30	27
cMet	-6.36835	-5.58479	-1.88318	55	0.064969	30	27
EGFR	-2.95785	-2.28105	-1.86840	55	0.067036	30	27
MTA1	-7.55365	-8.13656	1.81479	55	0.075011	30	27
RIZ1	-7.52785	-8.25903	1.79518	55	0.078119	30	27
ErbB3	-6.62488	-7.10826	1.79255	55	0.078545	30	27
TOP2B	0.54974	0.27531	1.74888	55	0.085891	30	27
EIF4E	-5.06603	-6.31426	1.68030	55	0.098571	30	27
TS	-2.95042	-2.36167	-1.67324	55	0.099959	30	27
STK15	-3.25010	-2.72118	-1.64822	55	0.105010	30	27

[0079] For each gene, a classification algorithm was utilized to identify the best threshold value (CT) for using each gene alone in predicting clinical outcome.

[0080] Based on the data set forth in Table 2, expression of the following genes in ER-positive cancer above a defined expression level is indicative of a reduced likelihood of survival without cancer recurrence following surgery: CD68; CTSL; FBXO5; SURV; CCNB1; MCM2; Chk1; MYBL2; HIF1A; cMET; EGFR; TS; STK15. Many of these genes (CD68, CTSL, SURV, CCNB1, MCM2, Chk1, MYBL2, EGFR, and STK15) were also identified as indicators of poor prognosis in the previous analysis, not limited to ER-positive breast cancer. Based on the data set forth in Table 2, expression of the following genes in ER-positive cancer above a defined expression level is indicative of a better prognosis for survival without cancer recurrence following surgery: IGFR1; BC12; HNF3A; TP53BP2; GATA3; BBC3; RAD51C; BAG1; IGFBP2; PR; CD9; RB1; EPHX1; CEGP1; TRAIL; DR5; p27; p53; MTA; RIZ1; ErbB3; TOP2B; EIF4E. Of the latter genes, IGFR1; BC12; TP53BP2; GATA3; BBC3; RAD51C; BAG1; IGFBP2; PR; CD9; CEGP1; DR5; p27; RIZ1; ErbB3; TOP2B; EIF4E have also been identified as indicators of good prognosis in the previous analysis, not limited to ER-positive breast cancer.

Analysis of ER negative patients by Binary approach

[0081] Twenty patients with normalized CT for estrogen receptor (ER) <1.6 (i.e., ER negative patients) were subjected to separate analysis. A t test was performed on the two groups of patients classified as either no recurrence and no breast cancer related death at three years, or recurrence or breast cancer-related death at three years, and the p-values for the differences between the groups for each gene were calculated. Table 3 lists the genes where the p-value for the differences between the groups was <0.118. The first column of mean expression values pertains to patients who neither had a metastatic recurrence nor died from breast cancer. The second column of mean expression values pertains to patients who either had a metastatic recurrence or died from breast cancer.

Table 3

	Mean	Mean	t-value	df	p	Valid N	Valid N
KRT14	-1.95323	-6.69231	4.03303	18	0.000780	5	15
KLK10	-2.68043	-7.11288	3.10321	18	0.006136	5	15
CCND1	-1.02285	0.03732	-2.77992	18	0.012357	5	15
Upa	-0.91272	-0.04773	-2.49460	18	0.022560	5	15
HNF3A	-6.04780	-2.36469	-2.43148	18	0.025707	5	15
Maspin	-3.56145	-6.18678	2.40169	18	0.027332	5	15
CDH1	-3.54450	-2.34984	-2.38755	18	0.028136	5	15
HER2	-1.48973	1.53108	-2.35826	18	0.029873	5	15
GRB7	-2.55289	0.00036	-2.32890	18	0.031714	5	15
AKT1	-0.36849	0.46222	-2.29737	18	0.033807	5	15
TGFA	-4.03137	-5.67225	2.28546	18	0.034632	5	15
FRP1	1.45776	-1.39459	2.27884	18	0.035097	5	15
STMY3	-1.59610	-0.26305	-2.23191	18	0.038570	5	15
Contig 27882	-4.27585	-7.34338	2.18700	18	0.042187	5	15
A-Catenin	-1.19790	-0.39085	-2.15624	18	0.044840	5	15
VDR	-4.37823	-2.37167	-2.15620	18	0.044844	5	15
GRO1	-3.65034	-5.97002	2.12286	18	0.047893	5	15
MCM3	-3.86041	-5.55078	2.10030	18	0.050061	5	15
B-actin	4.69672	5.19190	-2.04951	18	0.055273	5	15
HIF1A	-0.64183	-0.10566	-2.02301	18	0.058183	5	15
MMP9	-8.90613	-7.35163	-1.88747	18	0.075329	5	15
VEGF	0.37904	1.10778	-1.87451	18	0.077183	5	15
PRAME	-4.95855	-7.41973	1.86668	18	0.078322	5	15
AIB1	-3.12245	-1.92934	-1.86324	18	0.078829	5	15
KRT5	-1.32418	-3.62027	1.85919	18	0.079428	5	15
KRT18	1.08383	2.25369	-1.83831	18	0.082577	5	15
KRT17	-0.69073	-3.56536	1.78449	18	0.091209	5	15
P14ARF	-1.87104	-3.36534	1.63923	18	0.118525	5	15

[0082] Based on the data set forth in Table 3, expression of the following genes in ER-negative cancer above a defined expression level is indicative of a reduced likelihood of survival without cancer recurrence (p<0.05): CCND1; UPA; HNF3A; CDH1; Her2; GRB7; AKT1; STMY3; α-Catenin; VDR; GRO1. Only 2 of these genes (Her2 and Grb7) were also identified as indicators of poor prognosis in the previous analysis, not limited to ER-negative breast cancer. Based on the data set forth in Table 3, expression of the following genes in ER-negative

cancer above a defined expression level is indicative of a better prognosis for survival without cancer recurrence (KT14; KLK10; Maspin, TGF α , and FRP1. Of the latter genes, only KLK10 has been identified as an indicator of good prognosis in the previous analysis, not limited to ER-negative breast cancer.

Analysis of multiple genes and indicators of outcome

[0083] Two approaches were taken in order to determine whether using multiple genes would provide better discrimination between outcomes.

[0084] First, a discrimination analysis was performed using a forward stepwise approach. Models were generated that classified outcome with greater discrimination than was obtained with any single gene alone.

[0085] According to a second approach (time-to-event approach), for each gene a Cox Proportional Hazards model (see, e.g. Cox, D. R., and Oakes, D. (1984), Analysis of Survival Data, Chapman and Hall, London, New York) was defined with time to recurrence or death as the dependent variable, and the expression level of the gene as the independent variable. The genes that have a p-value < 0.10 in the Cox model were identified. For each gene, the Cox model provides the relative risk (RR) of recurrence or death for a unit change in the expression of the gene. One can choose to partition the patients into subgroups at any threshold value of the measured expression (on the CT scale), where all patients with expression values above the threshold have higher risk, and all patients with expression values below the threshold have lower risk, or vice versa, depending on whether the gene is an indicator of bad (RR>1.01) or good (RR<1.01) prognosis. Thus, any threshold value will define subgroups of patients with respectively increased or decreased risk. The results are summarized in Table 4. The third column, with the heading: exp(coef), shows RR values.

Table 4

Gene	coef	exp(coef)	se(coef)	z	p
TP53BP2	-0.21892	0.803386	0.068279	-3.20625	0.00134
GRB7	0.235697	1.265791	0.073541	3.204992	0.00135
PR	-0.10258	0.90251	0.035864	-2.86018	0.00423
CD68	0.465623	1.593006	0.167785	2.775115	0.00552
Bcl2	-0.26769	0.765146	0.100785	-2.65603	0.00791
KRT14	-0.11892	0.887877	0.046938	-2.53359	0.0113
PRAME	-0.13707	0.871912	0.054904	-2.49649	0.0125
CTSL	0.431499	1.539564	0.185237	2.329444	0.0198
EstR1	-0.07686	0.926018	0.034848	-2.20561	0.0274
Chk1	0.284466	1.329053	0.130823	2.174441	0.0297
IGFBP2	-0.2152	0.806376	0.099324	-2.16669	0.0303
HER2	0.155303	1.168011	0.072633	2.13818	0.0325
BAG1	-0.22695	0.796959	0.106377	-2.13346	0.0329
CEGP1	-0.07879	0.924236	0.036959	-2.13177	0.033
STK15	0.27947	1.322428	0.132762	2.105039	0.0353
KLK10	-0.11028	0.895588	0.05245	-2.10248	0.0355
B.Catenin	-0.16536	0.847586	0.084796	-1.95013	0.0512
EstR1	-0.0803	0.922842	0.042212	-1.90226	0.0571
GSTM1	-0.13209	0.876266	0.072211	-1.82915	0.0674
TOP2A	-0.11148	0.894512	0.061855	-1.80222	0.0715
AIB1	0.152968	1.165288	0.086332	1.771861	0.0764
FHIT	-0.15572	0.855802	0.088205	-1.7654	0.0775
RIZ1	-0.17467	0.839736	0.099464	-1.75609	0.0791
SURV	0.185784	1.204162	0.106625	1.742399	0.0814
IGF1	-0.10499	0.900338	0.060482	-1.73581	0.0826
BBC3	-0.1344	0.874243	0.077613	-1.73163	0.0833
IGF1R	-0.13484	0.873858	0.077889	-1.73115	0.0834
DIABLO	0.284336	1.32888	0.166556	1.707148	0.0878
TBP	-0.34404	0.7089	0.20564	-1.67303	0.0943
p27	-0.26002	0.771033	0.1564	-1.66256	0.0964
IRS1	-0.07585	0.926957	0.046096	-1.64542	0.0999

[0086] The binary and time-to-event analyses, with few exceptions, identified the same genes as prognostic markers. For example, comparison of Tables 1 and 4 shows that 10 genes were represented in the top 15 genes in both lists. Furthermore, when both analyses identified the same gene at [p<0.10], which happened for 21 genes, they were always concordant with respect to the direction (positive or negative sign) of the correlation with survival/recurrence. Overall, these results strengthen the conclusion that the identified markers have significant prognostic value.

[0087] For Cox models comprising more than two genes (multivariate models), stepwise entry of each individual gene into the model is performed, where the first gene entered is preselected from among those genes having significant univariate p-values, and the gene selected for entry into the model at each subsequent step is the gene that best improves the fit of the model to the data. This analysis can be performed with any total number of genes. In the analysis the results of which are shown below, stepwise entry was performed for up to 10 genes.

[0088] Multivariate analysis is performed using the following equation:

$$RR = \exp[\text{coef}(\text{geneA}) \times \text{Ct}(\text{geneA}) + \text{coef}(\text{geneB}) \times \text{Ct}(\text{geneB}) + \text{coef}(\text{geneC}) \times \text{Ct}(\text{geneC}) + \dots]$$

[0089] In this equation, coefficients for genes that are predictors of beneficial outcome are positive numbers and coefficients for genes that are predictors of unfavorable outcome are negative numbers. The "Ct" values in the equation are Δ Cts, i.e. reflect the difference between the average normalized Ct value for a population and the normalized Ct measured for the patient in question. The convention used in the present analysis has been that Δ Cts below and above the population average have positive signs and negative signs, respectively (reflecting greater or lesser mRNA abundance). The relative risk (RR) calculated by solving this equation will indicate if the patient has an enhanced or reduced chance of long-term survival without cancer recurrence.

Multivariate gene analysis of 79 patients with invasive breast carcinoma

[0090] A multivariate stepwise analysis, using the Cox Proportional Hazards Model, was performed on the gene expression data obtained for all 79 patients with invasive breast carcinoma. The following ten-gene sets have been identified by this analysis as having particularly strong predictive value of patient survival:

- (a) TP53BP2, Bcl2, BAD, EPHX1, PDGFR β , DIABLO, XIAP, YB1, CA9, and KRT8.
- (b) GRB7, CD68, TOP2A, Bcl2, DIABLO, CD3, ID1, PPM1D, MCM6, and WISP1.
- (c) PR, TP53BP2, PRAME, DIABLO, CTSL, IGFBP2, TIMP1, CA9, MMP9, and COX2.
- (d) CD68, GRB7, TOP2A, Bcl2, DIABLO, CD3, ID1, PPM1D, MCM6, and WISP1.
- (e) Bcl2, TP53BP2, BAD, EPHX1, PDGFR β , DIABLO, XIAP, YB1, CA9, and KRT8.
- (f) KRT14, KRT5, PRAME, TP53BP2, GUS1, AIB1, MCM3, CCNE1, MCM6, and ID1.
- (g) PRAME, TP53BP2, EstR1, DIABLO, CTSL, PPM1D, GRB7, DAPK1, BBC3, and VEGFB.
- (h) CTSL2, GRB7, TOP2A, CCNB1, Bcl2, DIABLO, PRAME, EMS1, CA9, and EpCAM.
- (i) EstR1, TP53BP2, PRAME, DIABLO, CTSL, PPM1D, GRB7, DAPK1, BBC3, and VEGFB.
- (k) Chk1, PRAME, p53BP2, GRB7, CA9, CTSL, CCNB1, TOP2A, tumor size, and IGFBP2.
- (l) IGFBP2, GRB7, PRAME, DIABLO, CTSL, β -Catenin, PPM1D, Chk1, WISP1, and LOT1.
- (m) HER2, TP53BP2, Bcl2, DIABLO, TIMP1, EPHX1, TOP2A, TRAIL, CA9, and AREG.
- (n) BAG1, TP53BP2, PRAME, IL6, CCNB1, PAI1, AREG, tumor size, CA9, and Ki67.
- (o) CEGP1, TP53BP2, PRAME, DIABLO, Bcl2, COX2, CCNE1, STK15, and AKT2, and FGF18.
- (p) STK15, TP53BP2, PRAME, IL6, CCNE1, AKT2, DIABLO, cMet, CCNE2, and COX2.
- (q) KLK10, EstR1, TP53BP2, PRAME, DIABLO, CTSL, PPM1D, GRB7, DAPK1, and BBC3.
- (r) AIB1, TP53BP2, Bcl2, DIABLO, TIMP1, CD3, p53, CA9, GRB7, and EPHX1.
- (s) BBC3, GRB7, CD68, PRAME, TOP2A, CCNB1, EPHX1, CTSL GSTM1, and APC.
- (t) CD9, GRB7, CD68, TOP2A, Bcl2, CCNB1, CD3, DIABLO, ID1, and PPM1D.
- (w) EGFR, KRT14, GRB7, TOP2A, CCNB1, CTSL, Bcl2, TP, KLK10, and CA9.
- (x) HIF1 α , PR, DIABLO, PRAME, Chk1, AKT2, GRB7, CCNE1, TOP2A, and CCNB1.
- (y) MDM2, TP53BP2, DIABLO, Bcl2, AIB1, TIMP1, CD3, p53, CA9, and HER2.
- (z) MYBL2, TP53BP2, PRAME, IL6, Bcl2, DIABLO, CCNE1, EPHX1, TIMP1, and CA9.
- (aa) p27, TP53BP2, PRAME, DIABLO, Bcl2, COX2, CCNE1, STK15, AKT2, and ID1.
- (ab) RAD51, GRB7, CD68, TOP2A, CIAP2, CCNB1, BAG1, IL6, FGFR1, and TP53BP2.
- (ac) SURV, GRB7, TOP2A, PRAME, CTSL, GSTM1, CCNB1, VDR, CA9, and CCNE2.
- (ad) TOP2B, TP53BP2, DIABLO, Bcl2, TIMP1, AIB1, CA9, p53, KRT8, and BAD.
- (ae) ZNF217, GRB7, p53BP2, PRAME, DIABLO, Bcl2, COX2, CCNE1, APC4, and β -Catenin.

[0091] 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.

Gene Accession Seq

AIB1	NM_006534	CGCGCGAGTTTCCGATTAAAGCTGAGCTGCGAGGAAATGGCGGCCGGGAGGATCAAATAC TTGCTGGATGGTGGACTCA
AKT1	NM_005163	CGCTTCATATGGCGCTGAGATTGTGTACGCCCTGGACTACCTGCACCTCGGAGAAGAACGTGGTGTACCGGGA
AKT2	NM_001626	TCCTGCCACCCCTTCAAACCTCAGGTCACGTCGAGGTCGACACAAGGTACTTCGATGATGAATTTACCGCC
APC	NM_000038	GGACAGCAGGAATGTGTTTCTCCATACAGGTCACGGGAGCCAAATGGTTTCAGAAACAAATCGAGTGGGT
AREG	NM_001657	TGTGAGTGAATGCCCTCTAGTAGTGAACCGCTCCTCGGGAGCCGACTATGACTACTCAGAAGATGATAACGAACCACAA
B-actin	NM_001101	CAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGCCCCCTCCATCGTCCACCGCAA TGC
B-Catenin	NM_001904	GGCTCTTGTGCGTACTGTCTTCGGGCTGGTGACAGGGAAGACATCACTGAGCTTGCCATCTGTGCTCTTCGTCTCTGA
BAD	NM_032989	GGGTCAGGTGCCCTCGAGATCGGGCTTGGGCCAGAGCATGTTCAGATCCCAGAGTTTGAGCCGAGTGAGCAG
BAG1	NM_004323	CGTGTGTCAGCACTTGGAAATACAAGATGGTTGCCGGGTCAATGTTAAATGGGAAAAAGAACAGTCCACAGGAAGAGGTTGAAC
BBC3	NM_014417	CCTGGAGGGTCCTGTACAATCTCATCATGGGACTCCTCTGCCCTTACCCAGGGCCACAGAGCCCCGAGATGGAGCCCAATTAG
Bcl2	NM_000633	CAGATGGACCTAGTACCCACTGAGATTTCACGCCGAAGGACAGCGATGGGAAAAATGCCCTTAATCATAGG
CA9	NM_001216	ATCCTAGCCCTGGTTTTTGGCCCTCCTTTTGTGTCACACAGCGTCGCGTTCTTGTGTCAGATGAGAAGGCAG
CCNB1	NM_031966	TTACAGTTGTGTCAGGAGACCATGTACATGACGTGCTCCATTATTGATCGGTTTCAAGCAGAATAATTGTGTCGCCAAGAAGATG
CCND1	NM_001758	GCATGTTCTGGGCTCTAAGATGAAGGAGACCATCCCCTGACGGCCGAGAAGCTGTGTCATCTACACCG
CCNE1	NM_001238	AAAGAAGATGATGACCGGGTTTACCCAAACTCAACGTGCAAGCCTCGGATTATTGCACCATCCAGAGGCTC
CCNE2	NM_057749	ATGCTGTGGCTCCTTCCTAAC TGGGGCTTTCTTGACATGTAGGTTGCTTGTTGAATAACCTTTTGTATATCACAAATTTGGGT
CD3z	NM_000734	AGATGAAGTGGAAAGGCGCTTTTCACCGCGGCCATCCTGCAGGCCACAGTTGCCGATTACAGAGGCA
CD68	NM_001251	TGGTTCACAGCCCTGTGTCCACCTCCAAGCCAGATTTCAGATTGAGTCATGTACACAACCCAGGGTGGAGGAG
CD9	NM_001769	GGGCGTGGAACAGTTTATCTCAGACATCTGCCCCAAAGAAGGACGTACTCGAAACCTTCACCGTG

CDH1	NM_004360	TGAGTGTCCCCCGTATCTTCCCCGCCCTGCCAATCCCGATGAAATTGGAAATTTTATTGATGAAAACTGAAAGCGGGCTG
CEGP1	NM_020974	TGACAAATCAGCACACCTGCATTACCCGCTCGGAAGAGGGCCTGAGCTGCATGAAATAGGATCACGGCTGTAGTCACA
Chk1	NM_001274	GATAAAATGGGTACAAGGGATCAGCTTTTCCCAGCCACATGTCTGATCATATGCTTTTGAATAGTCAGTTACTTGGCACCC
CIAP1	NM_001166	TGCCGTGTGGTGGGAAGCTCAGTAAC TGGGAACCAAAGGATGATGCTATGTGAGAACACCGAGGCATTTTCC
cIAP2	NM_001165	GGATATTTCCGTTGGCTCTTATTTCAAACCTCCATCAAATCTGTAAACTCCAGAGCAAAATCAAGATTTTTC TGCCTTGATGAGAA
cMet	NM_000245	GACATTTCCAGTCC TGCAGTCAATGCCCTCTCTGCCCCACCCCTTTGTTCAGTGTGGCTGGTGCCACGACAAATGTGTGCATCGGGAG
Contig 278	AK000618	GGCATCTGGCCCCAAGTTTCCCAAATCCAGCGCGCTAGAGGCCCATGCTTCCCAACTACCAGCTGAGGGGGTCT
COX2	NM_000963	CTGTCAGAGTTGGAAGCACTCTATGGTGACATCGATGGTGTGGAGCTGTATCCTGCCCTTCTGGTAGAAAAGCCTCGGC
CTSL	NM_001912	GGGAGGCTTATCTCACTGAGTGAGCAGAACTCGGTAGACTGCTCTGGGCCCAAGGCAATGAAAGGCTGCAATGG
CTSL2	NM_001333	TGTCTCACTGAGCGAGCAGAACTCGGTGGACTGTTCCGCTCTCAAGGCAATCAGGGCTGCAATGGT
DAPK1	NM_004938	CGCTGACATCATGAATGTTCC TCGACCCGGCTGGAGGCGAGTTTGGATATGACAAAGACACATCTGTGCTGAAAGAGA
DIABLO	NM_019887	CACAATGGCGGCTCTGAAGAGTTGGCTGTGCGCGAGCGTAACCTTATTCTTCAGGTACAGACAGTGTTTGTGT
DRS	NM_003842	CTCTGAGACAGTGTCTCGATGACTTTGCAGACTTGGTGCCCTTTGACTCCTGGGAGCCGCTCATGAGGAAGTTGGGCCCTATGG
EGFR	NM_005228	TGTCGATGGACTTCCAGAACCACCTGGGCAGCTGCCAAAAGTGTGATCCAAAGCTGTCCCAAT
EIF4E	NM_001968	GATCTAAGATGGCGACTGTGGAACCGGAAACCAACCCCTACTCCTAAATCCCGGACTACAGAAGAGGAGAAAACGGAATCTAA
EMS1	NM_005231	GGCAGTGTCACTGAGTCTTGAAATCTTCCCTGCCCGCGGGTCTCTGGATTGGGACGCACAGTGCA
EpCAM	NM_002354	GGGCCCTCCAGAACAATGATGGGCTTTATGATCTGACTGCGATGAGAGCGGGCTCTTTAAGGCCAAGGCCAAGCAGTGCA
EPHX1	NM_000120	ACCGTAGGCTCTGCTCTGAATGACTCTCTCTGTGGGTCTGGCTGCCATATTTCTAGAGAAGTTTTCACCTGGACCA
ErbB3	NM_001982	CGGTATATGTCATGCCAGATACACACCTCAAAGGTACTCCCTCCCTCCCGGGAAGGCACCCCTTTCCTCAGTGGGTCTCAGTTC
EstR1	NM_00125	CGTGGTGCCCTCTATGACCTGCTGCTGGAGATGCTGGAGCCACCCGCTTACATGCGCCCACTAGCC
FBXO5	NM_012177	GGGTATTCCTCATTTTCTCTACAAGATGGCTCTCAGTGAACATGAAGAAGGTAGCCTCTGGAGGAGAAATTCGGTGACAGTCTACAATCC
FGF18	NM_003862	CGGTAGTCAAGTCCGGATCAAGGGCAAGGAGACGGAATTCATCTGTGATGAACCGCAAGGCAAGC
FGFR1	NM_023109	CACGGGACATTCACCACATCGACTACTATAAAAAAGACAACCAACGGCCGACTGCCCTGTGAAGTGGATGGCACCC
FHIT	NM_002012	CCAGTGGAGCGCTTCCATGACCTGCGTCTGATGAAGTGGCCGATTGTTCAGACGACCCAGAGAG
FRP1	NM_003012	TTGGTACCTGTGGGTTAGCATCAAGTCTCCCCAGGGTAGAATTCATACAGAGCTCCAGTTTGCAATTTGGATGTG
G-Catenin	NM_002230	TCAGCAGCAAGGGCATCATGAGAGGAGATGAGGCC TCGGGCGCCAGTACACGCTCAAGAAAACCCACC
GAPDH	NM_002046	ATTCCACCCATGGCAAAATTCATGGCACCGTCAAGGCTGAGAACGGGAAGCTTGTCATCAATGGAATCCCATC
GATA3	NM_002051	CAAAGGAGCTCACTGTGGTGTCTGTGTTCCAACCACTGAATCTGGACCCCATCTGTGAATAAGCCATTC TGACTC
GRB7	NM_005310	CCATCTGCATCCATCTTTGTTGGGCTCCCCACCCCTTGAGAAGTGCC TCAGATAATACCC TGGTGGCC
GRO1	NM_001511	CGAAAAGATGCTGAACAGTGACAAATCCAAC TGACCAGAGGGAGGAGGAAGCTCACTGGTGGCTGTTCTGTA
GSTM1	NM_000561	AAGCTATGAGGAAAAGAAGTACACGATGGGGGACGCTCCTGATTATGACAGAAAGCCAGTGCTGTAATGAAAAATTCAGCTGGGCC
GUS	NM_000181	CCCACTCAGTAGCCAAAGTCAAAATGTTTGGAAAAACAGCCCGTTTACTTGAGCAAGACTGATACCACCTGCCGTG
HER2	NM_004448	CGGTGTGAGAAGTGCAGCAAGCCCTGTGCCCCAGTGTGCTATGGTCTGGGCA TGGAGCAC TTCCGAGAGG
HIF1A	NM_001530	TGAACATAAAGTCTGCAACATGGAAGGTATTGCAC TGACAGGCCACATTACGTATATGATACCAACCAACCACTCA
HNF3A	NM_004496	TCCAGGATGTTAGGAAC TGTGAAGATGGAAGGGCATGAAACCAGCGACTGGAACAGCTACTACGACAGACGCG
ID1	NM_002165	AGAACCAGCAAGGTGAGCAAGGTGGAGATTC TCCAGCACGTCA TCGACTACATCAGGGACCTTCAGTTGGA
IGF1	NM_000618	TCCGGAGCTGTGATCTAAGGAGGCTGGAGATGTA TTGCGCACCCCTCAAGCCTGCCAAGTCAGCTCGCTCTGTCCG
IGF1R	NM_000875	GCATGGTAGCCGAAGATTTACAGTCAAAAATCGGAGATTTTGGTATGACGCGAGATATCTATGAGACAGACTATTACCGGAAA
IGFBP2	NM_000597	GTGGACAGCACCATGAACATGTTGGGCGGGGGAGGCAGTGTGCGCCGGAAGCCCTCAAGTCCGGTATGAAGG
IL6	NM_000600	CCTGAACCTTCCAAAGATGGCTGAAAAAGATGGATGCTTCCAATCTGGATTCAATGAGGAGACTTGCCTGGT
IRS1	NM_005544	CCACAGCTCACTTCTGTCAAGTGTCCATCCAGCTCCAGCCAGCTCCACAGAGAGGAAGAGACTGGCAC TGAGG
KI-67	NM_002417	CGGACTTTGGGTGCCACTTGACGAGCGGTGGTTCGACAAGTGGCTTTGCGGGCCGATCGTCCAGTGGAGAGTTGTAA
KLK10	NM_002776	GCCCAGAGGCTCCATCGTCCATCTCTTCTCCCAAGTCGGCTGAACCTCTCCCTTTGCTGCACTGTTCAAACCTCTG
KRT14	NM_000526	GGCCTGCTGAGATCAAAGACTACAGTCCCTACTTCAAGACCATGAGGACCTGAGGAACAAGATTC TCACAGCCACAGTGGAC
KRT17	NM_000422	CGAGGATTGGTTCTTCAGCAAGACAGAGGAAC TGAACCGCAGGTGGCCACCAACAGTGAGCTGGTGCAGAGT
KRT18	NM_000224	AGAGATCGAGGCTCTCAAGGAGGAGCTGCTCTTCATGAAGAAGAACCACGAAGAGGAAGTAAAAGGCC
KRT19	NM_002276	TGAGCGGCAGAAATCAGGAGTACCAGCGGCTCATGGACATCAAGTCGCGGCTGGAGCAGGAGA TTGCCACCTACCGCA
KRT5	NM_000424	TCAGTGGAGAAGGAGTTGGACCAAGTCAACATCTCTGTTGTCACAGCAGTGTTCCTCTGGATATGGCA
KRT8	NM_002273	GGATGAAGCTTACA TGAACAAGGTAGAGCTGGAGTCTCGCC TGGAAAGGCTGACCGACGAGATCAACTTCTCAGGCAGCTATATG
LOT1 varia	NM_002656	GGAAAGACCACCTGAAAAACCACCTCCAGACCCACGACCCCAACAAAATGGCC TTTGGGTGTGAGGAGTGTGGGAAGAAGTAC
Maspin	NM_002639	CAGATGGCCACTTTGAGAACATTTTAGCTGACAAACAGTGTGAACGACCAGACCAAAATCCTTGTGGTTAATGCTGCC
MCM2	NM_004526	GACTTTTGCCCGCTACCTTTCATTCGGCGGTGACAACAATGAGCTGTTGCTCTTCATACTGAAGCAGTTAGTGGC
MCM3	NM_002388	GGAGAACAATCCCC TTGAGACAGAAATATGGCCTTTCTGTCTACAAGGATCACCAGACCATCACCATCCAGGAGAT
MCM6	NM_005915	TGATGGTCCATGTGTCTACATTCATCACAGGTTTCATACCAACACAGGC TTCAGCACTTCTTTGGTGTGTTTCTGCTGCCA
MDM2	NM_002392	CTACAGGGAGGCCATCGAA TCCGGATCTTGATGCTGGTGTAAAGTGAACATTTCAGGTGATTGGTTGGAT
MMFP9	NM_004994	GAGAACCAATCTCACCAGACAGCAGCTGGCAGAGGAATACCTGTACCCTATGGTTACACTCGGTG
MTA1	NM_004689	CCGCCCTCACTTGAAGAGAAACGCGCTCTTGGCGGACACTGGGGGAGGAGAGGAAGAAGCGCGGCTAACTTATTCC
MYBL2	NM_002466	GCCGAGATCGCCAAGATGTTGCCAGGGAGGACAGACAATGCTGTGAAGAATCACTGGAACCTACCATCAAAAG
P14ARF	S79535	CCCCTGTGCTGATGCTACTGAGGAGCCAGCGCTAGGGCAGCAGCCGCTTCC TAGAAGACCAGGTGATGATG
p27	NM_004064	CGGTGGACCACGAAGAGTTAACC CGGACTTGGAGAAGCACTGCAGAGACA TGAAGAGGCGAGCC
P53	NM_000546	CTTTGAACCTTGTCTTGCAATAGGTGTGCGTCAAGAAGCACCCAGGACTTCCATTTGCTTTGTCCCGG
PAI1	NM_000602	CCGCAACGTGGTTTTCTACCCCTATGGGGTGGCC TCGGTGTTGGCCA TGCTCCAGCTGACAACAGGAGGAGAAACCCAGCA

PDGFRb	NM_002609	CCAGCTCTCCTCCAGCTACAGATCAATGTCCCTGTCCGAGTGCTGGAGCTAAGTGAGAGCCACCC
PI3KC2A	NM_002645	ATACCAATCACCGCACAAACCCAGGCTATTGTGAAGTCCAGTCACAGCGCAAAGAAACATA TCGGGAGAAAATGCTAGTGTG
PPM1D	NM_003620	GCCATCCGCAAGGCCTTTCGCTTGTACCTTGCCATGTGGAAGAACTGGCGGAATGGCC
PR	NM_000926	GCATCAGGCTGTCAATTATGGTGCTTACCTGTGGGAGCTGTAAAGTCTCTTTAAGAGGGCAATGGAAGGGCAGCACAACTACT
PRAME	NM_006115	TCCTCATATCTGCCCTTGACAGAGTCTCCGACAGCACCATCGGGCTGAGCAATCTGACCCACGCTGC
pS2	NM_003225	GCCTCCCAAGTGTGCAATAAGGGCTGCTGTTTCGACGACACCGTTCGTGGGGTCCCTCGTGTCTTATCCATAATACCATCGAGC
RAD51C	NM_058216	GAACCTCTTGACGAGGAGCA TACCAGGGCTTCAATAACCTCTGTACAGCTAGATGATATCTGGGGTGGA
R81	NM_000321	CGAAGCCCTTACAGTTTCCTAGTTCACCCCTACGGATCCGAGGAAACATCTATATTTACCCCTGAAGAGTCC
RIZ1	NM_012231	CCAGACGAGCGATTAGAAGCGGCAGCTTGTGAGGTGAATGA TTTGGGGAAGAGGAGGAGGAGGAAGAGGAGGA
STK15	NM_003600	CATCTTCCAGGAGGACCACCTCTCTGTGGCACCCCTGGACTACCTGCCCCCTGAAATGATTGAAGGTCCGA
STMY3	NM_005940	CCTGGAGGCTGCAACATACCTCAATCCTGTCCAGGCCGGATCCTCTGAAGCCCTTTTCGAGCACTGCTATCTCCAAAGCCATTGTA

Table 5B

SURV	NM_001168	TGTTTGTATCCCGGGCTTACCAGGTGAGAAAGTGAGGGAGGAAGAAGGCAGTGTCCCTTTTGCTAGAGCTGACAGCTTTG
TBP	NM_003194	GCCCGAAACGCCGAATAATAATCCCAAGCGGTTTGCTGCGTAATCATGAGGATAAGAGGCCACG
TGFA	NM_003236	GGTGTGCCACAGACCTTCTTACTTTGGCTGTAAATCACTGTGCAGCCCTTTTGTGGGCTTCAAAACTGTCAAGAACTCCGT
TIMP1	NM_003254	TCCCTGCGGTCCAGATAGCCTGAATCCTGCCCGGAGTGAAGCTGAAGCCTGCACAGTGTCCACCCCTGTTCCAC
TOP2A	NM_001067	AATCCAAGGGGAGAGTGATGACTTCCATATGGACTTTGACTCAGCTGTGGCTCCTCGGGCAAAATCTGTAC
TOP2B	NM_001068	TGTGGACATCTTCCCTCAGACTTCCCTACTGAGCCACCCTTCTGCCACGAACCCGGTCCGGCTAG
TP	NM_001953	CTATATGCAGCCAGAGATGTGACAGCCACCGTGGACAGCCTGCCACTCATCACAGCCTCCATTCTCAGTAAGAACTCGTGG
TP53BP2	NM_005426	GGGCCAAATATTAGAAAGCTTTTATATCAGAGGACCACCATAGCGGCCATGGAGACCATCTCTGTCCCATCATACCCATCC
TRAIL	NM_003810	CTTCACAGTGTCTTGCAGTCTCTCTGTGTGGCTGTAACTTACGTGTACTTTACCAACGAGCTGAAGCAGATG
TS	NM_001071	GCCTCGGTGTGCCCTTTCAACATCGCCAGCTACGCCCTGCTCAGCTACATGATTGGCGCACATCAG
Upa	NM_002658	GTGGATGTGCCCTGAAGGACAAGCCAGGCGTCTACACGAGAGTCTACACTTCTTACCCCTGGATCCGAG
VDR	NM_000376	GCCTGGATTTCAGAAAGAGCCAAAGTCTGGATCTGGGACCCCTTCTCTCCCTGGCTGGTGTGAACT
VEGF	NM_003376	CTGCTGTCTTGGGTGCAATTGGAGCCCTTGCCTTGTCTTACCCTCCACCATGCCAAGTGGTCCAGGCTGC
VEGFB	NM_003377	TGACGATGGCCTGGAGTGTGTGCCACTGGGCAGCACCAAGTCCGGATGACAGATCCCTCATGATCCGGTACC
WISP1	NM_003882	AGAGGCATCCATGAACCTCACACTTGGCGGCTGCATCAGCACACGCTCCTATCAACCCAAGTACTGTGGAGTTTG
XIAP	NM_001167	GCAGTTGGAAGACACAGGAAAGTATCCCAAAATGTCAGATTTATCAACGGCTTTTATCTTGAATAAGTGCCACGCA
YB-1	NM_004559	AGACTGTGGAGTTTGATGTTGTTGAAGGAGAAAAGGTGCGGAGGCAGCAAAATGTACAGGTCCCTGGTGGTGTTC
ZNF217	NM_006526	ACCCAGTAGCAAGGAGAAGCCCACTCACTGCTCCGAGTGGCGCAAGGCTTTTCAAGACCTACCACCAAGCTG

Gene	Accession	Probe Name	Seq	Len
AIB1	NM_006534	S1994/AIB1.f3	GCGGCGAGTTTCCGATTTA	19
AIB1	NM_006534	S1995/AIB1.r3	TGAGTCCACCATCCAGCAAGT	21
AIB1	NM_006534	S5055/AIB1.p3	ATGGCGGCGGGAGGATCAAAA	21
AKT1	NM_005163	S0010/AKT1.f3	CGCTTCTATGGCGCTGAGAT	20
AKT1	NM_005163	S0012/AKT1.r3	TCCCGGTACACCAGTCTT	20
AKT1	NM_005163	S4776/AKT1.p3	CAGCCCTGGACTACCTGCACTCGG	24
AKT2	NM_001626	S0828/AKT2.f3	TCCTGCCACCCCTTCAAACC	19
AKT2	NM_001626	S0829/AKT2.r3	GGCGGTAATTCATCATCGAA	21
AKT2	NM_001626	S4727/AKT2.p3	CAGGTACGTCGAGGTCGACACA	24
APC	NM_000038	S0022/APC.f4	GGACAGCAGGAATGTGTTTC	20
APC	NM_000038	S0024/APC.r4	ACCCACTCGATTTGTTTCTG	20
APC	NM_000038	S4888/APC.p4	CATTGGCTCCCCGTGACCTGTA	22
AREG	NM_001657	S0025/AREG.f2	TGTGAGTGAATGCTTCTAGTAGTA	27
AREG	NM_001657	S0027/AREG.r2	TTGTGGTTCGTTATCATACTCTCTGA	27
AREG	NM_001657	S4889/AREG.p2	CCGTCCCTCGGGAGCCGACTATGA	23
B-actin	NM_001101	S0034/B-acti.f2	CAGCAGATGTGGATCAGCAAG	21
B-actin	NM_001101	S0036/B-acti.r2	GCATTTCGCTGGACGAT	18
B-actin	NM_001101	S4730/B-acti.p2	AGGAGTATGACGAGTCCGGCCCC	23
B-Catenin	NM_001904	S2150/B-Cate.f3	GGCTCTGTGCGTACTGTCTT	22
B-Catenin	NM_001904	S2151/B-Cate.r3	TCAGATGACGAAGAGCACAGATG	23
B-Catenin	NM_001904	S5046/B-Cate.p3	AGGCTCAGTGATGTCTTCCCTGTCACCAG	29
BAD	NM_032989	S2011/BAD.f1	GGGTCAGGTGCTCGAGAT	19
BAD	NM_032989	S2012/BAD.r1	CTGCTCACTCGGCTCAAACCTC	21
BAD	NM_032989	S5058/BAD.p1	TGGGCCCAGAGCATGTTCAGATC	24
BAG1	NM_004323	S1386/BAG1.f2	CGTTGTCAGCACTTGAATACAA	23
BAG1	NM_004323	S1387/BAG1.r2	GTCAACCTCTTCCCTGTGGACTGT	24
BAG1	NM_004323	S4731/BAG1.p2	CCCAATTAACATGACCCGGCAACCAT	26
BAG1	NM_014417	S1584/BBC3.f2	CCTGGAGGGTCCGTGTACAAT	20
BBC3	NM_014417	S1585/BBC3.r2	CTAATTGGGCTCCATCTCG	19
BBC3	NM_014417	S4890/BBC3.p2	CATCATGGGACTCCTGCCCTTACC	24
Bcl2	NM_000633	S0043/Bcl2.f2	CAGATGGACCTAGTACCCACTGAGA	25
Bcl2	NM_000633	S0045/Bcl2.r2	CCTATGATTTAAGGGCAATTTTCC	24
Bcl2	NM_000633	S4732/Bcl2.p2	TTCCACGCCGAAGGACAGCGAT	22
CA9	NM_001216	S1398/CA9.f3	ATCCTAGCCCTGGTTTTTGG	20

Gene	Accession	Probe Name	Seq	Len
CA9	NM_001216	S1399/CA9.r3	CTGCCCTTCTCATCTGCACAA	20
CA9	NM_001216	S4938/CA9.p3	TTTGCTGTCAACCAGCGTCGC	20
CCNB1	NM_031966	S1720/CCNB1.f2	TTCAGGTTGTTGCAGGAGAC	20
CCNB1	NM_031966	S1721/CCNB1.r2	CATCTTCTTGGGCACACAAT	20
CCNB1	NM_031966	S4733/CCNB1.p2	TGTCTCCATTATTGATCGGTTTCATGCA	27
CCND1	NM_001758	S0058/CCND1.f3	GCATGTTCTGGCCTCTAAGA	21
CCND1	NM_001758	S0060/CCND1.r3	CGGTGTAGATGCACAGCTTCTC	22
CCND1	NM_001758	S4986/CCND1.p3	AAGGAGACCATCCCCCTGACGGC	23
CCNE1	NM_001238	S1446/CCNE1.f1	AAAGAAGATGATGACCGGTTTAC	24
CCNE1	NM_001238	S1447/CCNE1.r1	GAGCCTCTGGATGGTGCAAT	20
CCNE1	NM_001238	S4944/CCNE1.p1	CAAACCAACGTGCAAGCCTCGGA	24
CCNE2	NM_057749	S1458/CCNE2.f2	ATGCTGTGGCTCCTTCCTAACT	22
CCNE2	NM_057749	S1459/CCNE2.r2	ACCCAAATTGTGATATACAAAAAGGTT	27
CCNE2	NM_057749	S4945/CCNE2.p2	TACCAAGCAACCTACATGTCAAGAAAGCCC	30
CD3z	NM_000734	S0064/CD3zf1	AGATGAAGTGGAAAGCGCTT	20
CD3z	NM_000734	S0066/CD3zf1	TGCCCTCTGTAATCGGCAACTG	21
CD3z	NM_000734	S4988/CD3zp1	CACCGCGGCCCATCCTGCA	18
CD68	NM_001251	S0067/CD68.f2	TGGTCCCCAGCCCTGTGT	18
CD68	NM_001251	S0069/CD68.r2	CTCCTCCACCCTGGGTTGT	19
CD68	NM_001251	S4734/CD68.p2	CTCCAAGCCCAGATTCAGATTCGAGTCA	28
CD9	NM_001769	S0686/CD9.f1	GGGCGTGGAAACAGTTTATCT	20
CD9	NM_001769	S0687/CD9.r1	CACGGTGAAGGTTTCGAGT	19
CD9	NM_001769	S4792/CD9.p1	AGACATCTGCCCCAAGAAGGACGT	24
CDH1	NM_004360	S0073/CDH1.f3	TGAGTGTCCCCCGGTATCTTC	21
CDH1	NM_004360	S0075/CDH1.r3	CAGCCGCTTTTCAGATTTTCAT	21
CDH1	NM_004360	S4990/CDH1.p3	TGCCAATCCCGATGAAAATTGAAAATT	27
CEGP1	NM_020974	S1494/CEGP1.f2	TGACAAATCAGCACACCTGCAT	21
CEGP1	NM_020974	S1495/CEGP1.r2	TGTGACTACAGCCGTGATCCTTA	23
CEGP1	NM_020974	S4735/CEGP1.p2	CAGGCCCTCTTCCGAGCGGT	20
Chk1	NM_001274	S1422/Chk1.f2	GATAAATTGGTACAAGGGATCAGCTT	26
Chk1	NM_001274	S1423/Chk1.r2	GGGTGCCAAGTAAGTACTATTCA	24
Chk1	NM_001274	S4941/Chk1.p2	CCAGCCACATGTCTGATCATATGC	26
CIAP1	NM_001166	S0764/CIAP1.f2	TGCCCTGTGGTGGGAAGCT	18
CIAP1	NM_001166	S0765/CIAP1.r2	GGAAAAATGCCCTCCGGTGT	19
CIAP1	NM_001166	S4802/CIAP1.p2	TGACATAGCATCATCCTTTGGTCCCAGTT	30
cIAP2	NM_001165	S0076/cIAP2.f2	GGATATTCCGTGGCTCTTATTCA	24
cIAP2	NM_001165	S0078/cIAP2.r2	CTTCTCATCAAGGCAGAAAAATCTT	25
cIAP2	NM_001165	S4991/cIAP2.p2	TCTCCATCAAAATCTGTAAACTCCAGAGCA	30
cMet	NM_000245	S0082/cMet.f2	GACATTTCCAGTCTGCAGTCA	22
cMet	NM_000245	S0084/cMet.r2	CTCCGATCGACACATTGT	20
cMet	NM_000245	S4993/cMet.p2	TGCCCTCTCTGCCCCACCCCTTGT	23
Contig 27882	AK000618	S2633/Contig.f3	GGCATCCTGGCCCAAAGT	18
Contig 27882	A000618	S2634/Contig.r3	GACCCCTCAGCTGGTAGTTG	21
Contig 27882	AK000618	S4977/Contig.p3	CCCAAATCCAGGCGCTAGAGGC	23
COX2	NM_000963	S0088/COX2.f1	TCTGCAGAGTTGGAAGCACTCTA	23
COX2	NM_000963	S0090/COX2.r1	GCCGAGGCTTTCTACCAGAA	21
COX2	NM_000963	S4995/COX2.p1	CAGGATACAGCTCCACAGCATCGATGTC	28
CTSL	NM_001912	S1303/CTSL.f2	GGGAGGCTTATCTCACTGAGTGA	23
CTSL	NM_001912	S1304/CTSL.r2	CCATTGCAGCCTTCATTGC	19
CTSL	NM_001912	S4899/CTSL.p2	TTGAGGCCAGAGCAGTCTACCAGATTCT	29
CTSL2	NM_001333	S4354/CTSL2.f1	TGTCTCACTGAGCGAGCAGAA	21
CTSL2	NM_001333	S4355/CTSL2.r1	ACCATTGCAGCCCTGATTG	19
CTSL2	NM_001333	S4356/CTSL2.p1	CTTGAGGACGCGAACAGTCCACCA	24
DAPK1	NM_004938	S1768/DAPK1.f3	CGCTGACATCATGAATGTTCTT	22
DAPK1	NM_004938	S1769/DAPK1.r3	TCTCTTTCAGCAACGATGTGTCTT	24
DAPK1	NM_004938	S4927/DAPK1.p3	TCATATCCAACTCGCTCCAGCCG	25
DIABLO	NM_019887	S0808/DIABLO.f1	CACAATGGCGGCTCTGAAG	19
DIABLO	NM_019887	S0809/DIABLO.r1	ACACAAACACTGTCTGTACCTGAAGA	26
DIABLO	NM_019887	S4813/DIABLO.p1	AAGTTACGCTGCGCGACAGCCAA	23
DR5	NM_003842	S2551/DR5.f2	CTCTGAGACAGTCTTCGATGACT	24
DR5	NM_003842	S2552/DR5.r2	CCATGAGGCCCAACTTCCT	19
DR5	NM_003842	S4979/DR5.p2	CAGACTTGGTGCCCTTTGACTCC	23
EGFR	NM_005228	S0103/EGFR.f2	TGTGATGGACTTCCAGAAC	20

Gene	Accession	Probe Name	Seq	Len
EGFR	NM_005228	S0105/EGFR.r2	ATTGGGACAGCTTGATCA	19
EGFR	NM_005228	S4999/EGFR.p2	CACCTGGGCAGCTGCCAA	18
EIF4E	NM_001968	S0106/EIF4E.f1	GATCTAAGATGGCGACTGTGCGAA	23
EIF4E	NM_001968	S0108/EIF4E.r1	TTAGATTCCGTTTTCTCTCTCTG	25
EIF4E	NM_001968	S5000/EIF4E.p1	ACCACCCTACTCCTAATCCCCGACT	27
EMS1	NM_005231	S2663/EMS1.f1	GGCAGTGTCACTGAGTCTTGA	22
EMS1	NM_005231	S2664/EMS1.r1	TGCACTGTGCGTCCCAAT	18
ELMS1	NM_005231	S4956/EMS1.p1	ATCCTCCCCGTGCCCCGCG	18
EpCAM	NM_002354	S1807/EpCAM.f1	GGGCCCTCCAGAACAATGAT	20
EpCAM	NM_002354	S1808/EpCAM.r1	TGCACTGTCTGGCCTTAAAGA	21
EpCAM	NM_002354	S4984/EpCAM.p1	CCGCTCTCATCGAGTCAGGATCAT	25
EPHX1	NM_000120	S1865/EPHX1.f2	ACCGTAGGCTCTGCTCTGAA	20
EPHX1	NM_000120	S1866/EPHX1.r2	TGGTCCAGGTGGAAAAC TTC	20
EPHX1	NM_000120	S4754/EPHX1.p2	AGGCAGCCAGACCCACAGGA	20
ErbB3	NM_001982	S0112/ErbB3.f1	CGGTATGTCTATGCCAGATACAC	23
ErbB3	NM_001982	S0114/ErbB3.r1	GAAGTGAAGCCACTGAAGAAAGG	24
ErbB3	NM_001982	S5002/ErbB3.p1	CCTCAAAGGTAATCTCCCTCTCCCGG	25
EstR1	NM_000125	S0115/EstR1.f1	CGTGGTCCCCCTCTATGAC	19
EstR1	NM_000125	S0117/EstR1.r1	GGCTAGTGGGCGCATGTAG	19
EstR1	NM_000125	S4737/EstR1.p1	CTGGAGATGCTGGACGCC	19
FBXO5	NM_012177	S2017/FBXO5.r1	GGATTGTAGACTGTCAACCGAAATTC	25
FBXO5	NM_012177	S2018/FBXO5.f1	GGCTATTCTCTATTTCTCTACAAAGTG	28
FBXO5	NM_012177	S5061/FBXO5.p1	CCTCCAGGAGGCTACCTTCTTCATGTTAC	30
FGF18	NM_003862	S1665/FGF18.f2	CGGTAGTCAAGTCCGGATCAA	21
FGF18	NM_003862	S1666/FGF18.r2	GCTTGCCCTTGCGGTTCA	18
FGF18	NM_003862	S4914/FGF18.p2	CAAGGAGACGGAATTTCTACCTGTGC	25
FGFR1	NM_023109	S0818/FGFR1.f3	CACGGGACATTCACCACATC	20
FGFR1	NM_023109	S0819/FGFR1.r3	GGGTGCCATCCACTTCACA	19
FGFR1	NM_023109	S4816/FGFR1.p3	ATAAAAGACAACCAACGGCCGACTGC	27
FHIT	NM_002012	S2443/FHIT.f1	CCAGTGGAGCGCTTCAT	18
FHIT	NM_002012	S2444/FHIT.r1	CTCTCTGGGTCTGTAACAA	22
FHIT	NM_002012	S2445/FHIT.p1	TCGGCCACTTCATCAGGACGCAG	23
FHIT	NM_002012	S4921/FHIT.p1	TCGGCCACTTCATCAGGACGCAG	23
FRP1	NM_003012	S1804/FRP1.f3	TTGGTACCTGTGGGTTAGCA	20
FRP1	NM_003012	S1805/FRP1.r3	CACATCCAAATGCAAACTGG	20
FRP1	NM_003012	S4983/FRP1.p3	TCCCCAGGGTAGAATTCATCAGAGC	26
G-Catenin	NM_002230	S2153/G-Cate.f1	TCAGCAGCAAGGGCATCAT	19
G-Catenin	NM_002230	S2154/G-Cate.r1	GGTGGTTTCTTGAGCGTGACT	23
G-Catenin	NM_002230	S5044/G-Cate.p1	GCCTCCGAGGCTCATCCT	19
GAPDH	NM_002046	S0374/GAPDH.f1	ATTCCACCCATGGCAAATTC	20
GAPDH	NM_002046	S0375/GAPDH.r1	GATGGGATTTCATTGATGACA	22
GAPDH	NM_002046	S4738/GAPDH.p1	CCGTTCTCAGCCTTGACGGTGC	22
GATA3	NM_002051	S0127/GATA3.f3	CAAAGGAGCTCACTGTGGTGTCT	23
GATA3	NM_002051	S0129/GATA3.r3	GAGTCAGAATGGCTTATTCACAGATG	26
GATA3	NM_002051	S5005/GATA3.p3	TGTTCCAACCACTGAATCTGGACC	24
GRB7	NM_005310	S0130/GRB7.f2	CCATCTGCATCCATCTTGTT	20
GRB7	NM_005310	S0132/GRB7.r2	GGCCACCAGGGTATTATCTG	20
GRB7	NM_005310	S4726/GRB7.p2	CTCCCCACCCTTGAGAAGTGCCT	23
GRO1	NM_001511	S0133/GRO1.f2	CGAAAAGATGCTGAACAGTGACA	23
GRO1	NM_001511	S0135/GRO1.r2	TCAGGAACAGCCACCAAGTGA	20
GRO1	NM_001511	S5006/GRO1.p2	CTTCTCTCTCCCTCTGGTCAAGTTGGAT	28
GSTM1	NM_000561	S2026/GSTM1.r1	GGCCACGCTTGAAATTTTCA	20
GSTM1	NM_000561	S2027/GSTM1.f1	AAGCTATGAGGAAAAGAAGTACACGAT	27
GSTM1	NM_000561	S4739/GSTM1.p1	TCAGCCACTGGCTTCTGTCTAATCAGGAG	30
GUS	NM_000181	S0139/GUS.f1	CCCACCTAGTAGCCAAAGTCA	20
GUS	NM_000181	S0141/GUS.r1	CACGCAAGTGTATCAGTCT	20
GUS	NM_000181	S4740/GUS.p1	TCAAGTAAACGGGCTGTTTTCCAAACA	27
HER2	NM_004448	S0142/HER2.f3	CGGTGTGAGAAGTGACAGCAA	20
HER2	NM_004448	S0144/HER2.r3	CCTCTCGCAAGTGTCCAT	19
HER2	NM_004448	S4729/HER2.p3	CCAGACCATAGCACACTCGGACAC	24
HIF1A	NM_001530	S1207/HIF1A.f3	TGAACATAAAGCTGCAACATGGA	24
HIF1A	NM_001530	S1208/HIF1A.r3	TGAGGTGGTTACTGTTGGTATCATATA	28
HIF1A	NM_001530	S4753/HIF1A.p3	TTGCACTGCACAGGCCACATTCAC	24

Gene	Accession	Probe Name	Seq	Len
HNF3A	NM_004496	S0148/HNF3A.f1	TCCAGGATGTTAGGAACTGTGAAG	24
HNF3A	NM_004496	S0150/HNF3A.r1	GCGTGTCGCGTAGTAGCTGTT	22
HNF3A	NM_004496	S5008/HNF3A.p1	AGTCGCTGGTTTCATGCCCTTCCA	24
ID1	NM_002165	S0820/ID1.f1	AGAACCCGAAGGTGAGCAA	19
ID1	NM_002165	S0821/ID1.r1	TCCAACTGAAGGTCCCTGATG	21
ID1	NM_002165	S4832/ID1.p1	TGGAGATTCTCCAGCACGTCATCCAC	26
IGF1	NM_000618	S0154/IGF1.f2	TCCGGAGCTGTGATCTAAGGA	21
IGF1	NM_000618	S0156/IGF1.r2	CGGACAGAGCGAGCTGACTT	20
IGF1	NM_000618	S5010/IGF1.p2	TGTATTGCGCACCCCTCAAGCCTG	24
IGF1R	NM_000875	S1249/IGF1R.f3	GCATGGTAGCCGAAGATTCA'	21
IGF1 R	NM_000875	S1250/IGF1R.r3	TTCCGGTAATAGTCGTCTCATAGATATC	30
IGF1R	NM_000875	S4895/IGF1R.p3	CGCGTCATACCAAAATCTCCGATTTTGA	28
IGFBP2	NM_000597	S1128/IGFBP2.f1	GTGGACAGCACCATGAACA	19
IGFBP2	NM_000597	S1129/IGFBP2.r1	CCTTCATACCCGACTTGAGG	20
IGFBP2	NM_000597	S4837/IGFBP2.p1	CTTCGGCCAGCACTGCCTC	20
IL6	NM_000600	S0760/IL6.f3	CCTGAACCTTCCAAAGATGG	20
IL6	NM_000600	S0761/IL6.r3	ACCAGGCAAGTCTCCTCATT	20
IL6	NM_000600	S4800/IL6.p3	CCAGATTGGAAGCATCCATCTTTTCA	27
IRS1	NM_005544	S1943/IRS1.f3	CCACAGCTACCTTCTGTCA	20
IRS1	NM_005544	S1944/IRS1.r3	CCTCAGTGCCAGTCTCTTCC	20
IRS1	NM_005544	S5050/IRS1.p3	TCCATCCCAGCTCCAGCCAG	20
KI-67	NM_002417	S0436/KI-67.f2	CGGACTTTGGGTGCGACTT	19
KI-67	NM_002417	S0437/KI-67.r2	TTACAACTCTCCACTGGGACGAT	24
KI-67	NM_002417	S4741/KI-67.p2	CCACTTGTGGAACACCCTCGT	23
KLK10	NM_002776	S2624/KLK10.f3	GCCCAGAGGCTCCATCGT	18
KLK10	NM_002776	S2625/KLK10.r3	CAGAGGTTTGAACAGTGCAGACA	23
KLK10	NM_002776	S4978/KLK10.p3	CCTCTTCCCTCCCAGTCGGCTGA	23
KRT14	NM_000526	S1853/KRT14.f1	GGCCTGCTGAGATCAAAGAC	20
KRT14	NM_000526	S1854/KRT14.r1	GTCCACTGTGGCTGTGAGAA	20
KRT14	NM_000526	S5037/KRT14.p1	TGTTCTCAGGTCTCAATGGTCTTG	26
KRT17	NM_000422	S0172/KRT17.f2	CGAGGATTGCTTTCAGCAA	21
KRT17	NM_000422	S0174/KRT17.r2	ATCTGCACCAGCTCACTGTTG	22
KRT17	NM_000422	S5013/KRT17.p2	CACCTCGCGGTTCACTTCTCTGT	24
KRT18	NM_000224	S1710/KRT18.f2	AGAGATCGAGGCTCTCAAGG	20
KRT18	NM_000224	S1711/KRT18.r2	GGCCTTTTACTTCCTCTTCG	20
KRT18	NM_000224	S4762/KRT18.p2	TGGTCTTCTTCATGAAGAGCAGCTCC	27
KRT19	NM_002276	S1515/KRT19.f3	TGAGCGGCAGAACTCAGGAGTA	21
KRT19	NM_002276	S1516/KRT19.r3	TGCGGTAGGTGGCAATCTC	19
KRT19	NM_002276	S4866/KRT19.p3	CTCATGGACATCAAGTCGCGCTG	24
KRT5	NM_000424	S0175/KRT5.f3	TCAGTGGAGAAGGAGTTGGA	20
KRT5	NM_000424	S0177/KRT5.r3	TGCCATATCCAGAGGAAACA	20
KRT5	NM_000424	S5015/KRT5.p3	CCAGTCAACATCTCTGTTGTCACAAGCA	28
KRT8	NM_002273	S2588/KRT8.f3	GGATGAAGCTTACATGAACAAGGTAGA	27
KRT8	NM_002273	S2589/KRT8.r3	CATATAGCTGCCGTGAGGAAGTTGAT	25
KRT8	NM_002273	S4952/KRT8.p3	CGTCGGTCAGCCCTTCCAGGC	21
LOT1 variant 1	NM_002656	S0692/LOT1 v.f2	GGAAAGACCACCTGAAAAACCA	22
LOT1 variant 1	NM_002656	S0693/LOT1 v.r2	GTACTTCTCCCACTCTCTACA	24
LOT1 variant 1	NM_002656	S4793/LOT1 v.p2	ACCCACGACCCCAACAAAATGGC	23
Maspin	NM_002639	S0836/Maspin.f2	CAGATGGCCACTTTGAGAACATT	23
Maspin	NM_002639	S0837/Maspin.r2	GGCAGCATTAAACCACAAGGATT	22
Maspin	NM_002639	S4835/Maspin.p2	AGCTGACAACAGTGTGAACGACCAGACC	28
MCM2	NM_004526	S1602/MCM2.f2	GACTTTTGCCCGCTACCTTTC	21
MCM2	NM_004526	S1603/MCM2.r2	GCCACTAACTGCTTCAGTATGAAGAG	26
MCM2	NM_004526	S4900/MCM2.p2	ACAGCTCATTTGTTGTACGCCCGGA	24
MCM3	NM_002388	S1524/MCM3.f3	GGAGAAACAATCCCTTGAGA	20
MCM3	NM_002388	S1525/MCM3.r3	ATCTCCTGGATGGTGATGGT	20
MCM3	NM_002388	S4870/MCM3.p3	TGGCCTTCTGTCTACAAGGATCACCA	27
MCM6	NM_005915	S1704/MCM6.f3	TGATGGTCTATGTGTCAATTCA	24
MCM6	NM_005915	S1705/MCM6.r3	TGGGACAGGAAACACACCAA	20
MCM6	NM_005915	S4919/MCM6.p3	CAGGTTTCATACCAACACAGCCTCAGCAC	30
MDM2	NM_002392	S0830/MDM2.f1	CTACAGGGACGCCATCGAA	19
MDM2	NM_002392	S0831/MDM2.r1	ATCCAACCAATCACCCTGAATGTT	23
MDM2	NM_002392	S4834/MDM2.p1	CTTACACCAGCATCAAGATCCGG	23

Gene	Accession	Probe Name	Seq	Len
MMP9	NM_004994	S0656/MMP9.f1	GAGAACCAATCTCACCGACA	20
MMP9	NM_004994	S0657/MMP9.r1	CACCCGAGTGTAAACCATAGC	20
MMP9	NM_004994	S4760/MMP9.p1	ACAGGATTTCTCTGCCAGCTGCC	24
MTA1	NM_004689	S2369/MTA1.f1	CCGCCCTCACCTGAAGAGA	19
MTA1	NM_004689	S2370/MTA1.r1	GGAATAAGTAGCCGCGCTTCT	22
MTA1	NM_004689	S4855/MTA1.p1	CCCAGTGTCCGCCAAGGACG	21
MYBL2	NM_002466	S3270/MYBL2.f1	GCCGAGATCGCCAAGATG	18
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SEQUENCE LISTING

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<110> GENOMIC HEALTH, INC.
RUSH UNIVERSITY MEDICAL CENTER
COBLEIGH, Melody
SHIAK, Steven
BAKER, Joffre
CRONIN, Maureen

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REFERENCES CITED IN THE DESCRIPTION

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P A T E N T K R A V

1. Fremgangsmåde til forudsigelse af sandsynligheden for overlevelse på langt sigt hos en patient med brystkræft uden tilbagefald af brystkræft, som omfatter bestemmelse af et niveau af et RNA-transkript af MYBL2 i en brystkræft-
5 vævsprøve, der er tilvejebragt fra patienten;
normalisering af nævnte niveau af RNA-transkriptet for at opnå et normaliseret ekspressionsniveau af MYBL2;
hvor forøget normaliseret ekspressionsniveau af MYBL2 sammenlignet med det normaliserede niveau af RNA-transkriptet af MYBL2 i et brystkræftsvævs-referencesæt
10 indikerer en reduceret sandsynlighed for overlevelse på langt sigt uden tilbagefald af brystkræft.
2. Fremgangsmåde ifølge krav 1, som yderligere omfatter bestemmelse af et normaliseret ekspressionsniveau af et RNA-transkript af mindst ét gen udvalgt blandt GRB7, CD68, CTSL, Chkl, AIB1, CCNB1, MCM2, FBXO5, Her2, STK15, SURV, EGFR, HIF1α
15 og TS eller deres ekspressionsprodukter;
hvor forøgede normaliserede ekspressionsniveauer af det mindst ene gen indikerer reduceret sandsynlighed for overlevelse på langt sigt uden tilbagefald af brystkræft.
3. Fremgangsmåde ifølge krav 1, hvor brystkræften er invasivt brystkarcinom.
4. Fremgangsmåde ifølge krav 1, hvor brystkræft-tumorprøven er et fikseret,
20 voksindlejret vævseksempplar.
5. Fremgangsmåde ifølge krav 1, hvor brystkræften er østrogenreceptor-positiv brystkræft.
6. Fremgangsmåde ifølge krav 1, hvor MYBL2 RNA-transkriptniveauet bestemmes af kvantitativ revers transkriptase-polymerasekædereaktion (qRT-PCR).
- 25 7. Fremgangsmåde ifølge krav 1, som yderligere omfatter tilvejebringelse af en rapport baseret på det normaliserede ekspressionsniveau af MYBL2.
8. Fremgangsmåde ifølge krav 7, hvor rapporten indeholder en forudsigelse af sandsynligheden for patientens overlevelse på langt sigt uden tilbagefald af brystkræft.